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Insights in the immune response against *Escherichia coli* O157:H7 infection in sheep

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List of abbreviations

A/E	Attaching and effacing lesion
ABTS	2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) diammonium salt
AEC	3-amino-9-ethylcarbazole
ASC	Antibody-secreting cells
AUC	Area under the curve
BSA	Bovine serum albumin
C. rodentium	Citrobacter rodentium
C1-INH	C1 esterase inhibitor
CFU	Colony forming unit
Cif	Cycle inhibiting factor
CoLN	Colon lymph node
ConA	Concanavalin A
CPM	Counts per minute
DAEC	Diffusely adherent Escherichia coli
DNA	Deoxyribonucleic acid
Dpi	Days post inoculation
Dppi	Days post primary inoculation
E. coli	Escherichia coli
EAEC	Enteroaggregative Escherichia coli
Efa	EHEC factor of adherence
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic Escherichia coli
EIEC	Enteroinvasive Escherichia coli
ELISA	Enzym-linked immunosorbent assay
ELISPOT	Enzym-linked immunospot
EPEC	Enteropathogenic Escherichia coli
Esp	Escherichia coli secreted protein
ETEC	Enterotoxigenic Escherichia coli
FCS	foetal calf serum
Gb3	Globotriaosylceramide
Gb4	Globotetraosylceramide
HBSS	Hank's balanced salt solution
HRP	Horse-radish peroxidase
HUS	Haemolytic uremic syndrome
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IMS	Immunomagnetic separation
IPP	Ileal Peyer's patches
IRSp53	Insulin receptor substrate protein of 53 kDa
IRTKS	Insulin receptor tyrosine kinase substrate

JAK/STAT	Janus kinase/signal transducer and activator of transcription
JPP	Jejunal Peyer's patches
LEE	Locus of Enterocyte Effacement
LPJ	Lamina propria of jejunum
LPR	Lamina propria of rectum
LPS	Lipopolysaccharide
Map	Mitochondrion-associated protein
MC	Monomorphonuclear cells
MDa	Megadalton
MLN	Mesenterial lymph node
NalCT-SMAC	Sorbitol MacConkey agar supplemented with cefixime, tellurite and nalidixic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nle	Non-LEE encoded effectors
N-WASP	Neural Wiskott Aldrich syndrome protein
OD	Optical density
OmpA	Outer membrane protein A
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood monomorphonuclear cells
PBS	Phosphate buffered saline
RAJ	Recto-anal junction
RLN	Rectal lymph node
RNA	Ribonucleic acid
SEM	Standard error of the mean
SRP	Siderophore receptor and porin-protein
STEC	Shiga toxin-producing Escherichia coli
Stx	Shiga toxin
TccP	Tir-cytoskeleton coupling protein
TER	transepithelial electrical resistance
Tir	Translocated intimin receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
TTSS	Type III secretion system
VTEC	Verocytotoxic Eschericia coli

Part I: Literature review

1. Introduction

Escherichia coli (*E. coli*) is part of the commensal bacterial flora of the gastrointestinal tract of mammals and birds. Although *E. coli* lives in a symbiotic state with the host, some intestinal *E. coli* strains have acquired virulence factors that permit them to cause a wide range of enteric diseases. These pathotypes are found worldwide and pose serious medical and economical problems in developing and industrialized countries. The diarrhoeagenic *E. coli* strains are classified according to their virulence characteristics (Nataro and Kaper, 1998) (Figure 1).

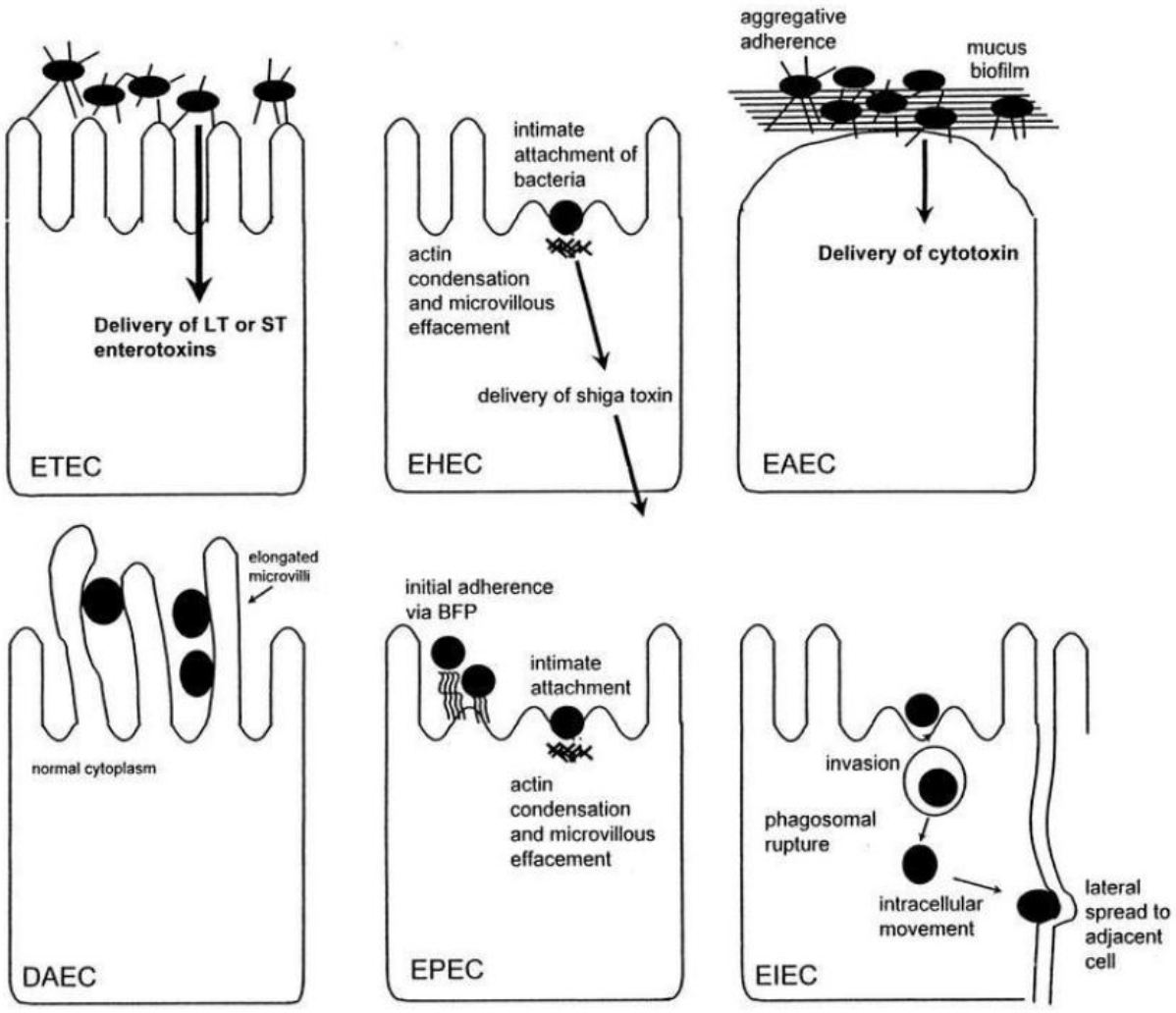


Figure 1: Overview of diarrhoeagenic *E. coli* (Adapted from Nataro and Kaper, 1998).

Enterotoxigenic *E. coli* (ETEC) is a major cause of travellers' diarrhoea, diarrhoea in children and post-weaning disease in piglets. ETEC colonizes the small intestine by means of fimbriae, proteinaceous surface structures that mediate attachment to the intestinal epithelium. Colonization is followed by production of heat-stable and/or heat-labile toxins. The action of these enterotoxins causes inhibition of sodium chloride absorption by villus tip cells, resulting in osmotic diarrhoea (Nataro and Kaper, 1998).

Enteropathogenic *E. coli* (EPEC) is a pathogen of the small intestine that causes severe watery diarrhoea, particularly among infants in developing countries. The hallmark of EPEC infection is the production of a typical histopathological lesion, the attaching and effacing (A/E) lesion. Bacteria attach firmly to the epithelial cells in the intestine, leading to local destruction of microvilli and formation of a pedestal underneath adherent bacteria (Knutton et al., 1989). The virulence factors responsible for this lesion are encoded on a pathogenicity island in the genome, the Locus of Enterocyte Effacement (LEE) (McDaniel et al., 1995). The most important of these factors is the *eae*-gene, encoding the intimin protein that mediates the intimate adhesion.

Enterohaemorrhagic *E. coli* (EHEC) is a subgroup of Shiga toxin-producing *E. coli* (STEC), also named verocytotoxin-producing *E. coli* (VTEC), characterized by the production of Shiga toxins (Stx). STEC have one or more *stx* genes but do not necessarily cause disease, whereas EHEC strains are pathogenic for humans. EHEC causes diarrhoea, haemorrhagic colitis and the potentially fatal haemolytic uremic syndrome (HUS). EHEC strains are related to EPEC, sharing the ability to produce A/E lesions. Although many STEC serotypes have been associated with human illness, *E. coli* O157:H7 is by far the most frequently implicated serotype in sporadic cases and outbreaks worldwide (Nataro and Kaper, 1998). Ruminants constitute the major reservoir (Karmali, 1989).

Enteroaggregative *E. coli* (EAEC) has been associated with acute and persistent diarrhoea in children, adults, travellers and immunocompromized individuals in developing and industrialized countries (Law and Chart, 1998). Where EPEC and EHEC adhere to intestinal epithelial cells in a localized manner, EAEC adhere in an aggregative, stacked brick pattern, with bacteria trapped in a mucus biofilm (Nataro et al., 1987). EAEC strains secrete entero- and cytotoxins, and induce mucosal inflammation (Nataro et al., 1995).

Diffusely adherent *E. coli* (DAEC) is a heterogeneous group of bacteria that attach to epithelial cells in a diffuse pattern, distinct from EAEC (Scaletsky et al., 1984). DAEC strains are associated with watery diarrhoea in children in different regions, but little is known about the pathogenic characteristics (Levine et al., 1993). DAEC can produce a wide range of adhesins, such as the family of afimbrial adhesins and the AIDA adhesin (Benz and Schmidt, 1989; Bilge et al., 1989; Le Bouguenec and Servin, 2006).

Enteroinvasive *E. coli* (EIEC) causes bacillary dysentery with high resemblance to the disease caused by the *Shigella* species (Nataro and Kaper, 1998). Bacteria invade the intestinal epithelial cells, followed by intracellular multiplication and dissemination to adjacent cells. Both *Shigella* and EIEC carry a large invasion plasmid and express a similar set of proteins. Infection is transmitted by direct contact from person to person or via contaminated food and water (Parsot, 2005).

All mentioned pathotypes cause diarrhoea and have a large arsenal of virulence factors to subvert host cellular functions. However, the distinction between the different pathotypes is not always clear and more specifically, the relationship between STEC, EHEC and EPEC is complex. EHEC is thought to have evolved from an EPEC ancestor, *E. coli* O55:H7, and has gained pathogenicity through the acquisition of Shiga toxins and other virulence factors encoded on transposable bacteriophage elements and plasmids (Whittam et al., 1993; Lathem et al., 2003). Nevertheless, EHEC strains are not simply EPEC strains that produce Shiga toxins. Although EPEC and EHEC adhere in a similar manner, the adhesion factors and the mechanisms of this adhesion are not identical (Garmendia et al., 2005; Bardiau et al., 2010). This is best illustrated by the occurrence of different *eae*-types in EPEC and EHEC strains, and in other A/E pathogens (Ramachandran et al., 2003; Lacher et al., 2006). Furthermore, EPEC and EHEC both insert their own receptor, the translocated intimin receptor (Tir), into the host cell membrane, but the subsequent events leading to the formation of the A/E lesion are different, as well as the reportoire and function of the inserted virulence factors (Garmendia et al., 2005; Tobe et al., 2006).

Additionally, the characterization of EHEC and STEC strains can be confusing. EHEC strains cause disease in humans and are best described as *stx*-positive and LEE-positive (as evidenced by the presence of the *eae*-gene) but also *eae*-negative STEC strains are known to cause HUS (Bonnet et al., 1998; Paton et al., 1999). On the other hand, it has been described that

EHEC strains can loose the ability to produce Shiga toxins during disease progression or during subculturing although they were initially isolated from patients diagnosed with EHEC-induced HUS (Schmidt et al., 1999; Bielaszewska et al., 2009).

This literature review focuses on *E. coli* O157:H7, although much knowledge is derived from research performed with STEC and EPEC strains, and even *Citrobacter rodentium (C. rodentium),* a related A/E mouse pathogen that functions as a model for human EHEC infection (Schauer and Falkow, 1993).

2. *E. coli* O157:H7 in humans

2.1. Pathogenesis

After having survived the acidic environment of the stomach, *E. coli* O157:H7 adheres to the intestinal epithelium leading to A/E lesion formation, destruction of microvilli and induction of inflammation. Colonization is followed by release of Stx, which leads to damage to the mucosal endothelium. It is still unclear how Stx produced in the intestinal lumen is transported to the small blood vessels. Probably, the toxin is translocated through the epithelial cells into the circulation (Acheson et al., 1996), although the presence of the Stx receptor has not been detected on human enterocytes. Inflammation of the colonic mucosa is seen in *E. coli* O157:H7-infected patients, and it has been proposed that mucosal damage generates a way for Stx to cross the epithelial barrier (Gobert et al., 2008). Once in the circulation, there is evidence that Shiga toxin is transported by polymorphonuclear leucocytes to the kidneys although this is the subject of debate (te Loo et al., 2000; Geelen et al., 2007). Subsequently, Stx is internalized in the target-cells via binding to its receptor globotriaosylceramide (Gb3) (Lingwood, 1993). The action of Stx on the endothelial cells of the small capillaries causes the characteristic haemorrhages. Furthermore, the concerted action of several virulence factors (discussed further below) causes extensive mucosal damage.

2.2. Clinical manifestations

The infectious dose for humans is presumably very low since an ingestion of less than 100 bacteria can cause an infection (Lin et al., 1996). After ingestion of the bacteria, there is an incubation period of typically 3-4 days. Infection with *E. coli* O157:H7 is characterized by the sudden onset of severe abdominal cramps and watery diarrhoea that progresses rapidly to bloody diarrhoea (haemorrhagic colitis). The diarrhoea usually lasts 1-8 days. Vomiting can

occur, but fever is often absent (Riley et al., 1983; Su and Brandt, 1995; Coia, 1998). The infection can be complicated by a potentially life-threatening illness, HUS. HUS is characterized by thrombocytopenia (low platelet counts), microangiopathic haemolytic anaemia (anaemia due to damaged blood vessels leading to destruction of red blood cells, causing fatigue and weakness) and acute kidney failure. Some patients with HUS also develop complications of nerve or brain damage, such as seizures or strokes. Patients often need dialysis and are at risk of developing long-term sequelae (Siegler et al., 1994), while 3-12% percent of HUS-patients die (Garg et al., 2003; Siegler, 2003). HUS typically presents a few days after the onset of acute diarrhoeal illness and affects especially young children, elderly people and immunocompromized patients (Karmali et al., 1985; Griffin et al., 1988; Tarr et al., 2005; Gould et al., 2009). HUS and haemorrhagic colitis are caused by the action of Stx released by *E. coli* O157:H7 on blood vessels in the kidney and the intestine. Treatment of *E. coli* O157:H7 infections includes rehydration therapy and is mainly symptomatic and supportive (Griffin et al., 1988; Tarr, 1995). Anti-motility agents are contra-indicated (Tarr and Neill, 2001). Use of antibiotics remains controversial as bacterial lysis can lead to increased Stx release (Kimmitt et al., 2000; Kohler et al., 2000; Tarr et al., 2005) but not all studies agree on the adverse effects of particular antibiotics (Grif et al., 1998; Shiomi et al., 1999; Panos et al., 2006).

2.3. Incidence

Since the initial reports (Riley et al., 1983), sporadic cases and outbreaks of *E. coli* O157:H7 infection have been increasingly reported, which can partly be explained by improved detection methods and increased surveillance. Worldwide, reported annual rates of *E. coli* O157:H7 infections vary greatly, with incidence numbers per 100,000 inhabitants of 4.6 cases in Scotland (Health protection Scotland, 2010), 1.5 in the U.S. (Gould et al., 2009) and 0.9 in Australia (McPherson et al., 2009). Rates in Scotland tend to be higher than in most other European and North-American countries (Pearce et al., 2009). Argentina has the highest worldwide incidence of reported HUS cases in children younger than five year of age, with 12.2 cases per 100,000 children (Rivas et al., 2008). Reported HUS rates are 1.42 cases per 100,000 children younger than 16 in Switzerland (Schifferli et al., 2009) and 4.3 per 100,000 children under 5 years of age in Belgium (Cornu et al., 1999). Most cases are sporadic, but larger outbreaks have often been associated with *E. coli* O157:H7, such as the spinach outbreak in 2006 in the U.S., with 238 infected persons (Centers for Disease Control and Prevention, 2009). The

largest *E. coli* O157:H7 outbreak occurred in Japan and involved more than 6,000 children on a total of 12,860 diagnosed cases (Michino et al., 1999). Although STEC infections mostly occur in developed countries, outbreaks have been reported taking place in African countries as well (Germani et al., 1997; Cunin et al., 1999; Effler et al., 2001).

According to the most recent available data from the European Food Safety Authority (EFSA) 3,159 cases of STEC infections occurred in 2008 in the European Union (EU) member states (Table 1). Fifty-three percent of these cases were caused by serotype O157:H7 (European Food Safety Authority, 2010).

Table 1: Confirmed STEC cases reported by European countries in 2008.

Country	**Incidence per 100,000 inhabitants**	**Cases**
Austria	0.8	69
Belgium	1.0	103
Bulgaria	0	0
Cyprus	0.3	2
Czech Republic	-	-
Denmark	2.9	161
Estonia	0.2	3
Finland	0.2	8
France	0.1	85
Germany	1.1	876
Greece	0	0
Hungary	0	0
Ireland	4.8	213
Italy	< 0.1	24
Latvia	0	0
Lithuania	0	0
Luxembourg	0.8	4
Malta	1.9	8
Netherlands	0.6	92
Poland	< 0.1	3
Portugal	-	-
Romania	< 0.1	4
Slovakia	0.1	8
Slovenia	0.3	7
Spain	< 0.1	21
Sweden	3.3	304
United Kingdom	1.9	1164
EU total	0.7	3159
Iceland	1.3	4
Liechtenstein	0	0
Norway	0.5	22
Switzerland	0.9	67

Adapted from EFSA (2010)

It is important to note that the reported data are likely an underestimation of the true incidence, since STEC infections are not notifiable in all countries, laboratory isolation methods differ between countries, not all laboratories screen routinely for STEC, and many infections pass unnoticed, or are mild, without the need for medical attention. Although the incidence is low compared to the two most important foodborne infections, *Campylobacter and Salmonella* (European Food Safety Authority, 2010), STEC infections and *E. coli* O157:H7 in particular, are considered important due to the severity of the illness and the high case-fatality rate (Coia, 1998).

3. Animal reservoir

3.1. Epidemiology

E. coli O157:H7 was first recognized as a foodborne pathogen after an outbreak of haemorrhagic colitis in the U.S. in 1982, caused by consumption of undercooked hamburgers (Riley et al., 1983). At the same time, the link between presence of Stx in faeces of patients and HUS was established (Karmali et al., 1983). Since then, the pathogen has been recognized as a zoonotic agent, spread by animals to humans by the faecal-oral route. Ever since the first human outbreak was linked with ground beef consumption a bovine reservoir was suspected. Indeed, cattle have been identified as the most important reservoir (Karmali, 1989). During slaughter, the bacteria can be transmitted from the hide and the intestines to the carcass, and may cause a foodborne infection when the meat is not sufficiently heated prior to consumption. This is not the only route of transmission, an *E. coli* O157:H7 infection can occur through consumption of a wide variety of contaminated foods, including unpasteurized juice, raw milk, and raw produce (e.g., lettuce, spinach, and alfalfa sprouts) (Caprioli et al., 2005) and even fermented products (Conedera et al., 2007). Outbreaks have been linked to contaminated drinking water or recreational water (Paunio et al., 1999; Bruce et al., 2003; Richards, 2005). Direct contact with animals on farms and on contaminated pastures have also resulted in outbreaks of infection (Caprioli et al., 2005) and person-to-person spread to family members or in child-care settings have caused secondary cases of infection (Al-Jader et al., 1999; Galanis et al., 2003). However, *E. coli* O157:H7 does not always result in clinical illness, as evidenced by the isolation of the bacteria from asymptomatic carriers (Griffin and Tauxe, 1991; Silvestro et al., 2004).

3.2. Prevalence

Cattle are considered to be the major reservoir of *E. coli* O157:H7, harbouring the bacteria in their gastrointestinal tract without developing clinical symptoms as seen in humans. Occasionally, *E. coli* O157:H7 has been associated with diarrhoea in young calves and diarrhoea can be induced by experimental infection with *E. coli* O157:H7 (Dean-Nystrom et al., 1997; Dean-Nystrom et al., 1999; Kang et al., 2004), but the susceptibility of calves to *E. coli* O157:H7 diarrhoea decreases with age (Mechie et al., 1997; Shinagawa et al., 2000; Nielsen et al., 2002).

Occurrence of *E. coli* O157:H7 is very common in cattle. Estimates of prevalence range from 0 to 71% of individual animals and from 0 to 100% of herds, and involve reports from countries worldwide (reviewed by Gyles, 2007). A recent study demonstrated a farm prevalence of 37.8% in Belgian cattle farms (Cobbaut et al., 2009), where the proportion of cattle being positive within a farm ranged from 0 to 85% (Cobbaut et al., 2008). In a large-scale study performed in Scotland from 1998 until 2000, 207 out of 952 sampled farms (21.7%) were found to be positive for *E. coli* O157:H7 (Pearce et al., 2009). A Dutch study based on sampling of 1051 dairy herds and 930 veal herds revealed a herd-level prevalence of 8 and 12% for dairy and veal herds respectively (Berends et al., 2008). In the U.K., the within-herd prevalence of *E. coli* O157:H7 ranged from 0 to 60% (mean 24%) when 30 groups of young cattle on 30 farms were observed for 7 months and sampled on 4 to 6 separate occasions (Ellis-Iversen et al., 2009). The overall prevalence of cattle at slaughter in Kansas, U.S., was 20.3%, determined by the presence of *E. coli* O157:H7 in at least one intestinal site (Walker et al., 2010). Seventy-one percent of cattle arriving in small-scale U.S. processing plants had *E. coli* O157:H7 on the hide, whereas 33% of the pre-evisceration carcasses were positive. Only 2% of these carcasses had *E. coli* O157:H7 levels higher than 0.8 CFU/100 cm^2 (Bosilevac et al., 2009). The contrast between the prevalence before and after slaughter is also evident in Argentina where 10.5% of the calves were positive compared to 2.6% of carcasses (Masana et al., 2010).

Not only cattle but small ruminants as well are a reservoir for *E. coli* O157:H7. The bacteria have been isolated from sheep (Kudva et al., 1996, 1997a), goats (Pritchard et al., 2000; Orden et al., 2010) and wild ruminants (Renter et al., 2001; Garcia-Sanchez et al., 2007; Sanchez et al., 2010). Prevalence rates for individual sheep based on examination of faecal samples varied from 0 to 7.3% in different studies conducted in Europe and the U.S. (Sutherland et al., 2009; Oporto et al., 2008; Franco et al., 2009; Solecki et al., 2009).

Apart from being isolated from ruminants, *E. coli* O157:H7 has also been found sporadically in a wide variety of animals including pigs, horses, poultry and companion animals (Trevena et al., 1996; Doane et al., 2007; Feder et al., 2007; Pritchard et al., 2009). These animals are generally not regarded as a reservoir but can be a natural source of *E. coli* O157:H7 and therefore pose a risk to the public health.

Literature searches reveal that reported prevalence rates vary greatly, and many factors can influence the results: the used detection method (direct culture or enrichment with immunomagnetic separation, the culture medium and agar plates, PCR-based detection methods,...), the type of samples (faecal samples, rectal swabs, environmental samples, hide or carcass swabs,...), the sampling method (single sample or longitudinal study), testing individual animals or testing at the herd level, age of the animals, the farm type, seasonal variation etc. Therefore, reported rates are probably an underestimation of the true prevalence of *E. coli* O157:H7. In addition, the duration of shedding of *E. coli* O157:H7 by naturally infected cattle is highly variable. The length of the observed shedding is influenced by the detection method, with sensitive methods more likely to detect longer periods of shedding. Longitudinal studies have shown that shedding of *E. coli* O157:H7 is transient (Smith et al., 2010). Three distinct patterns were reported by several authors: non-persistent shedding lasting not more than 7 days, moderately persistent shedding lasting approximately one month, or persistent shedding lasting several months to even a year (Rice et al., 2003; Robinson et al., 2004; Baines et al., 2008). Natural fluctuations within an animal over time may be caused by intrinsic factors such as gut flora, gut health, behaviour or other anatomical, physiological or physical stresses (Robinson et al., 2009) or by external factors. Indeed, the risk of detecting *E. coli* O157:H7 was almost five times higher when the cattle were housed indoors compared to cattle grazing of pasture (Ellis-Iversen et al., 2009). Influence of diet has been reported (reviewed by Jacob et al., 2009). Shedding also appears to be related to weaning and age. Lowest rates were reported in calves before weaning, highest rates occurred in the post-weaning period and levels in adult cattle were intermediate (Mechie et al., 1997; Shinagawa et al., 2000; Nielsen et al., 2002). The prevalence of *E. coli* O157:H7 is low in winter, increases during spring and peaks in the warm summer months (Hancock et al., 1997; Tutenel et al., 2002).

A small proportion of cattle shed high levels of *E. coli* O157:H7 and have great potential to transmit the bacteria to other animals in the herd. These so-called supershedders excrete more than 10^3 CFU/g, and do so for longer periods of time (Omisakin et al., 2003; Low et al.,

2005; Matthews et al., 2006b). It is unknown why certain animals become supershedders whereas others do not.

3.3. Survival of *E. coli* O157:H7 in the environment

Besides the production of Shiga toxins, the highly virulent character of *E. coli* O157:H7 is associated with the fact that this bacterium can survive in harsh conditions and can grow at temperatures between 6 and 45,5°C, in a pH range of 4,4 to 9,0 and water activities between 0,95 and 0,99 (Raghubeer and Matches, 1990; Clavero and Beuchat, 1996; Ferenc et al., 2000; Echeverry et al., 2006). Thus, *E. coli* O157:H7 excreted into the environment appears to be well adapted to survive in different matrices such as water, soil and animal faeces, for periods ranging from several weeks to several months (Echeverry et al., 2006; Ibekwe et al., 2007; Avery et al., 2008). *E. coli* O157 even has the capacity to survive and to replicate in different bedding materials in the presence of urine (Conedera et al., 2001; Davis et al., 2005; Lejeune and Kauffman, 2005). In a study performed in Belgium, samples from the pen floor were positive for *E. coli* O157 on six sampling occasions whereas the pathogen was not detected in the faeces from the animals staying in the pen (Cobbaut et al., 2008). Manure from cattle production facilities can contain viable *E. coli* O157:H7, thus providing a source of human infection by contamination of the water supply or produce when it is used as fertilizer or as irrigation water on crops (Callaway et al., 2009).

3.4. Tissue tropism

The observation that *E. coli* O157:H7 could hardly be isolated from contents along the gastrointestinal tract of naturally infected cattle despite being abundantly present in faeces from (10^3 to 10^5 CFU/g) has led to the discovery of the terminal rectum as a primary colonization site in cattle (Grauke et al., 2002; Naylor et al., 2003). The majority of tissue-associated bacteria were recovered from the recto-anal junction (RAJ) of persistently colonized cattle following natural and experimental infection (Naylor et al., 2003). In contrast, non-pathogenic *E. coli* were present throughout the gastrointestinal tract and were mostly associated with digesta rather than the mucosa (Laven et al., 2003; Naylor et al., 2003). Factors that drive the tropism for the rectum are mostly unknown. Naylor et al. (2003) suggested that the tropism for this site may be related to the presence of lymphoid follicles in the RAJ, since other members of the *Enterobacteriaceae*, e.g., *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, *Shigella* and *Yersinia*

species have well-described tropisms for Peyer's patches, another region of gut-associated lymphoid tissue and known induction sites of immune responses. However, Lim et al. (2007a) stated that this can not be the sole reason for the rectal tropism, as *E. coli* O157:H7 does not colonize other tissues rich in lymphoid tissue. Nevertheless, it is interesting that *E. coli* O157:H7 colonizes a site where it can interact immediately with the host immune system, especially since the bacterium is known to influence immune responses (discussed further).

More likely is a receptor-based tropism. There is evidence that intimin-γ, an important factor mediating adherence that is specific for *E. coli* O157:H7, shows more restricted tissue affinity than other intimin types (Hartland et al., 2000). Complementation of an intimin-γ mutant of *E. coli* O157:H7 with intimin-α from an EPEC strain resulted in altered tissue tropism, namely colonization of both the large and small intestine, as normally seen in EPEC (Tzipori et al., 1995). Intimin-γ binds not only to its specific receptor Tir that is inserted into the host membrane by the bacteria itself, but also binds to the host receptors β1-integrin and nucleolin (Sinclair et al., 2006). Differential expression of these receptors along the gastrointestinal tract could play a role in the rectal tropism but remains to be determined. Moreover, *E. coli* O157:H7 might be able to influence the expression of the host receptors at the terminal rectum: it is known that Stx2 can increase the expression of nucleolin on HEp-2 cells (Robinson et al., 2006). A second adhesion based hypothesis involves StcE, a metalloprotease secreted by *E. coli* O157:H7. Grys et al. (2005) have demonstrated that StcE promotes intestinal adherence by cleaving glycoproteins in the mucus layer, bringing the bacteria in closer contact with the cell surface. This could in turn lead to a reduction of commensal bacteria in the proximity, thereby providing an advantage for *E. coli* O157:H7 to compete for space and nutrients.

Besides receptor-based mechanisms, there could be a physiological and biochemical basis for the observed tissue tropism. Since the concentration of $NaHCO_3$ in the lower intestinal tract is relatively high compared to the concentration in the upper small intestine, the $NaHCO_3$ may be an important signalling factor for promoting colonization of EHEC in the lower intestinal tract of humans (Abe et al., 2002). Another possibility is a nutritional basis for the rectal tissue tropism: bovine intestinal mucins contain L-fucose, which could be a preferred nutrient for *E. coli* O157:H7. A mutant *E. coli* O157:H7 strain unable to catabolise L-fucose exhibited significantly less colonization than the wild type strain as measured in faecal and rectal mucus samples (Snider et al., 2009). Spatial differences in availability of L-fucose or other sugars might explain the rectal tissue tropism. The composition of bovine rectal mucins is yet unknown. Other

proposed mechanisms include differences in lower volatile fatty acid concentrations, higher pH, mucus production etc., leading to more favourable conditions for survival and growth of *E. coli* O157:H7 in the rectum compared to other intestinal sites (Fox et al., 2007).

Finally, an animal can harbour different *E. coli* O157:H7 strains and certain strains might be better adapted to colonization in the rectum than others, which merely pass the intestine and only cause a transient shedding (Fox et al., 2008).

A difficulty in determining the exact mechanism behind the rectal tissue tropism is the detection limit of adherent bacteria in histological sections. *E. coli* O157:H7 counts of 10^5 to 10^6 per cm^2 are deemed necessary for demonstrating bacterial adherence (Bertschinger et al., 1972; Nart et al., 2008b).

4. Options for the control of *E. coli* O157:H7 in ruminants

After the outbreak in 1982 was traced to contaminated hamburgers, it became obvious that meat should be sufficiently cooked before consumption to kill pathogenic bacteria that might be present. In recent years, it has become apparent that contaminated meat is far from the only source for *E. coli* O157:H7 infection, substantiating the need for efficient control strategies. Decontamination processes are implemented to reduce the pathogen burden on carcasses, and many other methods are studied to control *E. coli* O157:H7 in ruminants. Because *E. coli* O157:H7 is widespread on cattle farms throughout the world and it can be isolated from many animal species, complete eradication is not realistic. It has been calculated that pre-harvest interventions that reduce the prevalence of *E. coli* O157:H7 in faeces will lead to fewer human infections (Jordan et al., 1999). Moreover, the strategic targeting of supershedders would drastically reduce the transmission of *E. coli* O157:H7 between herds and within slaughterhouses (Matthews et al., 2006a).

4.1. Exposure reduction strategies

Fairly simple management practices on the farm and in slaughterhouses are important and have the potential to reduce the general bacterial contamination (LeJeune and Wetzel, 2007). Good management practices ensure hygienic conditions on the farm and can reduce the prevalence of *E. coli* O157:H7. These measures include providing clean pens, clean water troughs, and dry bedding to cattle, but also maintaining animals in the same group, keeping calves separate from older animals and excluding the cattle population from wildlife (LeJeune

and Wetzel, 2007; Ellis-Iversen et al., 2008). Visibly dirty animals and stables tend to have higher *E. coli* O157:H7 rates (Nastasijevic et al., 2008). On the other hand, an animal does not have to be intestinally colonized to have a contaminated hide or vice versa (Van Donkersgoed et al., 1997; Reid et al., 2002). Transfer of bacteria from one animal to another can occur during transport to the slaughterhouse, leading not only to contamination of more animals brought into the slaughterhouse, but also contamination of the transport vehicle (Arthur et al., 2007a; Cuesta Alonso et al., 2007). In the slaughterhouse, much of the bacterial contamination of the carcass occurs during removal of the hide (Madden et al., 2004). Good slaughtering practices like cleaning knives and washing hands in between carcasses may prevent further contamination. Decontamination measures such as washing procedures or the use of organic acids, are implemented on the carcasses, but have not always been shown to be effective (Bosilevac et al., 2006; Arthur et al., 2007b; Penney et al., 2007; Laury et al., 2009). Furthermore, chemical decontamination may lead to discolouration of the carcass, affecting the consumer's concern towards meat.

4.2. Feed management

Other strategies aim at modifying the gastrointestinal flora of ruminants to prevent establishment or to remove *E. coli* O157:H7. The type of feed (grain, hay) given to cattle appears to have an influence on prevalence and shedding (reviewed in Jacob et al., 2009). Berg et al. (2004) reported higher *E. coli* O157:H7 concentrations in cattle on a barley grain diet than in cattle on a corn-based diet, and shedding was decreased in sheep that were switched from a grass diet to an alfalfa diet (Kudva et al., 1997b). Following dietary changes or withholding feed, animals that appeared to be culture negative started excreting *E. coli* O157:H7 (Kudva et al., 1995). The mechanisms behind the effect of diet on *E. coli* O157:H7 prevalence is poorly understood but include changes in volatile fatty acid concentrations, pH changes in the rumen and altering the residual microflora (Jacob et al., 2009). Apart from the type of feed having an influence on the prevalence, the feed itself may be contaminated with *E. coli* O157:H7 (Lynn et al., 1998).

4.3. Probiotics

Probiotics are live, non-pathogenic micro-organisms that confer a health benefit to the host. Probiotics (also named "direct-fed microbials") have been used in the cattle industry for

over 20 years to enhance animal health and production, and are more recently investigated as a means to reduce foodborne pathogens in animals, including *E. coli* O157:H7. *Pseudomonas aeruginosa* strains inhibitory to *E. coli* O157:H7 have been isolated from the rumen of sheep (Duncan et al., 1999), and *E. coli* strains isolated from healthy cattle reduced the excretion of serotype O157:H7 in experimentally infected cattle by producing colicins (Zhao et al., 1998; Schamberger et al., 2004). *Lactobacillus* strains have shown considerable promise in reducing intestinal colonization and faecal shedding of *E. coli* O157:H7 in experimentally infected calves (Ohya et al., 2000) and sheep (Lema et al., 2001), and several large studies have confirmed the potential of probiotics to reduce EHEC shedding in weaned calves and harvest-ready feedlot cattle (Tkalcic et al., 2003; Younts-Dahl et al., 2005; Stephens et al., 2007) without affecting feedlot performance of the cattle (Brashears et al., 2003). *Lactobacillus* strains exert their beneficial effect through the production of lactic acid (Ogawa et al., 2001), increase of volatile fatty acid concentrations in cattle faeces (Ohya et al., 2000), reduction of biofilm formation of *E. coli* O157:H7 (Kim et al., 2009) and have even been shown to prevent the *E. coli* O157:H7-mediated inhibition of IFN-γ-induced transduction signals in epithelial cells (Jandu et al., 2009).

4.4. Bacteriophages

Bacteriophages are viruses that infect bacteria, inject their DNA and make use of the biosynthetic machinery of the bacterial host to produce daughter phages, which are released via host lysis in order to repeat the process in other target bacteria (Callaway et al., 2008). Bacteriophages specific for *E. coli* O157:H7 have been isolated from the faeces of cattle and sheep and were able to eliminate *E. coli* O157:H7 *in vitro* (Kudva et al., 1999). Bacteriophages have also shown their merit in the removal of *E. coli* O157:H7 contamination from various hard surfaces, fruits, vegetables and ground beef, with up to 100% reduction (Abuladze et al., 2008). However, *in vivo* studies demonstrate variable results in ruminants (Table 2). Two separate studies with O157-specific phages given orally in single or multiple doses showed that the phages were ineffective in reducing *E. coli* O157:H7 excretion in sheep (Bach et al., 2003; Sheng et al., 2006a), whereas application of another phage on the recto-anal mucosa of steers resulted in significantly lower levels of *E. coli* O157:H7 excretion during 10 days post treatment as compared to the control group (Sheng et al., 2006a). In contrast, the inhibitory effect of a phage cocktail was transient and was not effective at the RAJ. It has been proposed that phage cocktails might be more effective, but a mixture of four phages did not lead to significant

differences in reduction of *E. coli* O157:H7 excretion between control and treatment groups of cattle (Rozema et al., 2009). Results from two independent trials where phages were administered 2-3 days after experimental challenge with *E. coli* O157:H7, indicated that numbers of *E. coli* O157:H7 in faeces and/or intestinal contents of sheep were significantly reduced (1,5 to 3 $\log_{10}$ reduction) but suggest that the inhibitory effect is transient and may not have been effective at the RAJ, the preferred colonization site in cattle (Raya et al., 2006; Callaway et al., 2008). Although generally regarded as a safe, natural and non-antibiotic method to reduce pathogens from the food supply, bacteriophage therapy does not yet suffice to be commercially used in ruminants for elimination of *E. coli* O157:H7.

To date, bacteriophage therapy has not been tested in a natural infection setting.

Table 2: Bacteriophage treatment for control of *E. coli* O157:H7 in ruminants.

Animal species	***E. coli* O157:H7 inoculation**	**Phage treatment (type, route, administration)**	**Follow-up after phage treatment**	**Result**	**Reference**
Sheep	Oral	Phage DC22, oral, 2 days pi	27 days	No significant reduction	Bach et al., 2003
Sheep	Oral	Phage CEV1, oral, 3 days pi	2 days (slaughter)	2-3 $\log_{10}$ reduction in caecal and rectal, but not ruminal contents	Raya et al., 2006
Sheep	Oral	Phage KH1, oral, 1, 9, 10 and 11 days pi	21 days	No significant reduction	Sheng et al., 2006a
Steers	Rectal	2-phage-mix, rectal, 7, 8, 9 and 11 days pi + daily addition of phages in drinking water	10 days	1.3-1.5 $\log_{10}$ reduction from day 1 to day 10	Sheng et al., 2006a
Sheep	Oral	8-phage-mix, oral, 2 and 3 days pi	4 days (slaughter)	1.5 – 2 $\log_{10}$ reduction	Callaway et al., 2008
Steers	Oral	4-phage-mix, oral	83 days	No significant reduction	Rozema et al., 2009
Steers	Oral	4-phage-mix, rectal	83 days	No significant reduction	Rozema et al., 2009
Steers	Oral	4-phage-mix, oral and rectal	83 days	No significant reduction	Rozema et al., 2009

pi: post inoculation

4.5. Vaccination

Several research groups have attempted vaccination of cattle as a means to control intestinal colonization, with variable results (Table 3). A vaccine based on type III secreted proteins (TTSS) reduced *E. coli* O157:H7 prevalence in feedlot cattle, colonization at the terminal rectum and hide contamination, and induced herd immunity (Smith et al., 2008; Moxley et al., 2009; Smith et al., 2009a; Smith et al., 2009b). *Econiche™ (developed by* Bioniche Life Sciences Inc., Belleville, Ontario, Canada) received full licensing approval from the Canadian Food Inspection Agency in October 2008, holds a conditional license for the U.S. market, and is thereby the first commercially available *E. coli* O157:H7 vaccine for use in cattle. However, this vaccine is not capable of completely clearing the infection in cattle even after three vaccine doses. A completely different strategy targets the bacterial need for iron as nutrient. A siderophore receptor and porin-protein (SRP) based vaccine formulation disrupts the iron transport system, causing bacterial cell death, and effectively reduced the prevalence of *E. coli* O157:H7 in two cattle feedlot settings (Fox et al., 2009; Thomson et al., 2009). This vaccine received a conditional license for the U.S. market in 2009 (Epitopix LLC, Willmar, Minnesota, U.S). A conditional license indicates that additional experiments have to be performed to demonstrate the efficacy of the vaccine.

All the above mentioned methods are promising in reducing *E. coli* O157 prevalence, but it is unclear to what extent preharvest burden needs to be reduced to effectively prevent contamination of consumable products. The infectious dose to colonize a ruminant has been estimated to be lower than 300 CFU (Besser et al., 2001), indicating that levels should be drastically reduced to curtail transmission between animals. Interventions should be applied on different levels, i.e. on the farm, in the slaughterhouse, in the processing plants, and last but not least, the consumer should take minimal precautions. Only with a multi-level strategy can maximal reductions in bacterial load be achieved, ensuring a safe food chain from farm to fork.

Antigen	Amount (µg) per dose	Doses	Adjuvant	Setting	Number of animals	Outcome	Reference
H7	60	3	Quil A	Experimental	32	Number of colonized animals is reduced, but no effect on total shedding	McNeilly et al., 2008
H7	60	2	Quil A	Experimental	5	Systemically induced IgG possibly interferes with innate immunity and thereby reduces vaccine efficacy	McNeilly et al., 2010
Intimin, EspA and Tir ± H7	60 of each antigen	3	Quil A	Experimental	30	50-71% reduction in number of colonized animals, with a 1-4 $\log_{10}$ reduction in faecal shedding; addition of H7 increases protection	
Intimin, Efa-1	100	2	Alu-Oil[a] or CT-B	Experimental	36	Vaccine induces humoral immunity but does not protect against intestinal colonization	van Diemen et al., 2007
EspA	100	3	IFA	Experimental	8	Vaccine induces humoral immunity but does not protect against intestinal colonization	Dziva et al., 2007b
Epitopix							
SRP	N/A[b]	2	N/A[b]	Experimental	30	Antibody response, no significant reduction in shedding	Thornton et al., 2009
SRP	N/A[b]	2 or 3	N/A[c]	Feedlot	2,536	Lower *E. coli* O157:H7 prevalence among vaccinated animals, 3 doses is more efficient than 2-dose regimen, 98.2% reduction in faecal *E. coli* O157:H7 concentration	Thomson et al., 2009
SRP	N/A[b]	2 (2 or 3 ml)	N/A	Feedlot	60	Lower *E. coli* O157:H7 prevalence among vaccinated animals, 3 ml dose is more efficient than 2 ml dose, less supershedders	Fox et al., 2009

CT-B: cholera toxin B subunit
Efa-1: EHEC factor for adherence
IFA: incomplete Freund's adjuvant
N/A: not available
SRP: siderophore and porin proteins

[a] Aluminium hydroxide oil-based adjuvant (Intervet international BV, Boxmeer, The Netherlands)
[b] Produced according to U.S. patent 6,027,736: Active immunization using a siderophore receptor protein.
[c] Commercial adjuvant (possibly Emulsigen) (MVP Laboratories, Ralston, Nebraska, U.S).

Antigen	Amount (µg) per dose	Doses	Adjuvant	Setting	Number of animals	Outcome	Reference
Bioniche							Potter et al., 2004
TTSS	50 or 200	2 or 3	VSA3 (Emulsigen D)	Experimental and feedlot	256	Prevalence of *E. coli* O157:H7 in vaccinated pens (8.8%) is reduced compared to non-vaccinated pens (21.3%)	Van Donkersgoed et al., 2005
TTSS	50	2	Emulsigen	Feedlot	218 pens[d]	No effect of vaccination on prevalence, possibly due to formalin treatment of antigen	Peterson et al., 2007b
TTSS	N/A	1, 2 or 3	Emulsigen D	Feedlot	608	Efficacy of 1-, 2- or 3-dose regimen is 68, 66 and 73% respectively; observation of herd immunity effect	Peterson et al., 2007a
TTSS	66.1	3	Emulsigen D	Feedlot	288	Vaccinated cattle were 98.3% less likely to be colonized at the terminal rectal mucosa	Smith et al., 2008
TTSS	N/A[e]	2	Emulsigen D	Feedlot	20,556	Probability for environmental transmission of *E. coli* O157:H7 is reduced within vaccinated pens	Smith et al., 2009a
TTSS	N/A[e]	2	Emulsigen D	Feedlot	504	Vaccination reduces rectal colonization, faecal shedding and hide contamination	Smith et al., 2009a
TTSS	N/A[e]	2	N/A[e]	Feedlot	718	Vaccinated cattle are 92% less likely to be colonized than non-vaccinated cattle at the terminal rectal mucosa	Smith et al., 2009b
TTSS	N/A[e]	2 or 3	N/A[e]	Feedlot	480	3 doses are more efficient than 2 doses in reducing the prevalence	Moxley et al., 2009

N/A: not available
TTSS: type three secreted proteins
[d] number of cattle unknown
[e] Vaccine prepared by Bioniche Life Sciences

5. Virulence factors and pathogenic features of EHEC

5.1. Shiga toxin

Shiga toxins are the major virulence determinants of STEC strains. There are two types: Stx1, which is almost identical to the Shiga toxin of *Shigella dysenteriae* type 1, and Stx2, which shares 56% amino acid identity with Stx1 (Strockbine et al., 1986). Each type has different antigenic variants, which differ in their biological activity and association with disease. Variants of Stx1 are thought to be genetic variants without major consequences on the cell toxicity, whereas the Stx2 family is more diverse and represents different biological variants (Mainil and Daube, 2005). The most commonly described variants are presented in Table 4.

The nomenclature is sometimes confusing, making it difficult to distinguish the variants (Scheutz et al., 2001; Mainil and Daube, 2005; Persson et al., 2007). STEC strains can possess one or more variants of Stx1, Stx2 or both. Epidemiological studies have shown that Stx2 is associated with more severe human disease.

Table 4: Different types of Shiga toxins

Stx type	Characteristics	Reference
Stx1	Shiga toxin produced by STEC and almost identical to Stx produced by *Shigella dysenteriae* serotype 1	Strockbine et al., 1986
Stx1c	Variant of Stx1 that is found in ovine and caprine strains but not in bovine strains and in some *eae*-negative STEC; associated with mild diarrhoea or no symptoms	Zhang et al., 2002; Brett et al., 2003
Stx1d	A variant from Stx1 isolated from bovine and human strains; associated with asymptomatic infections.	Burk et al., 2003
Stx2	Prototype of non-Stx1 toxins; associated with severe disease in humans	Strockbine et al., 1986
Stx2c	Associated with diarrhoea and HUS in humans; common in bovine and ovine STEC	Schmitt et al., 1991; Friedrich et al., 2002
Stx2d	Associated with *eae*-negative STEC and mild disease in humans	Pierard et al., 1998; Friedrich et al., 2002
Stx2dact	Vero cell cytotoxicity is increased 10- to 1000-fold by elastase in intestinal mucus; strains with this toxin are highly virulent	Kokai-Kun et al., 2000
Stx2e	A variant responsible for oedema disease of pigs; rare in human disease and associated with mild diarrhoea or asymptomatic infections in humans	Gyles et al., 1988; Sonntag et al., 2005a
Stx2f	A variant frequently isolated from pigeon droppings; rare in human disease	Sonntag et al., 2005b
Stx2g	A variant isolated from bovine strains; to date not associated with human disease	Leung et al., 2003

Adapted from Gyles (2007).

The *stx* genes are located in the genome of lambdoid prophages and are expressed when the lytic cycle of the phages is activated (Herold et al., 2004). This activation is linked to induction of the bacterial SOS response, that occurs upon DNA damage. When certain antibiotics are administered, the SOS response is activated and more Stx are produced and released, leading to aggravated disease (Kimmitt et al., 2000).

The Shiga toxins are part of the AB_5 family of toxins, with an A-subunit responsible for the enzymatic activity, and a pentameric ring of B-subunits that bind to the cellular receptor. Stx bind to Gb3 receptors on epithelial and endothelial cells (Jacewicz et al., 1986; Lingwood, 1993). The Stx2e variant binds preferentially to globotetraosylceramide (Gb4) but can also bind Gb3 (DeGrandis et al., 1989). After binding to the receptor, Stx are internalized by clathrin-mediated endocytosis and retrogradely transported to the Golgi apparatus and the endoplasmatic reticulum (Sandvig et al., 1992). In the cytosol, the A-subunit is proteolytically cleaved into an A1- and an A2-fragment. The A2-fragment remains attached to the B-subunit pentamer, and the A1 is now catalytically active (Lea et al., 1999). It specifically removes an adenine residue from the 28S rRNA component of eukaryotic ribosomes, leading to inhibition of protein synthesis in the cell. Certain cell types go into apoptosis as a result of ribocytotoxic stress response (Smith et al., 2003) or due to signalling by Stx (Cherla et al., 2003).

The absence of disease in cattle is explained by the lack of vascular receptors for Stx (Pruimboom-Brees et al., 2000). However, receptors are present on cortical kidney cells and crypt epithelial cells of the small and large intestine (Pruimboom-Brees et al., 2000; Hoey et al., 2002). By targeting crypt cells Stx may affect epithelial development. Indeed, reduced epithelial shedding prolonged colonization in ruminants (Magnuson et al., 2000). Stx also bind to submucosal lymphoid cells suggesting a role for Stx in immunomodulation (Hoey et al., 2002). Receptor-positive cells were not all equally susceptible to Stx-mediated toxicity, owing to the presence of different isoforms of Gb3 with differential sensitivities to Stx. In bovine primary colonic epithelial cells, Stx is transported into lysosomes instead of to the cytosol, leading to degradation of Stx (Hoey et al., 2003). Recently, it has been reported that epithelial and mesenchymal cells in the bovine colonic mucosa differ in their responsiveness to Stx1 (Stamm et al., 2008). Bovine epithelial cells resisted the cytolethal effect of Stx1 and did not respond to the toxin by releasing immune mediators. Mucosal macrophages carry Stx-receptors and alter their chemokine and cytokine expression patterns in response to Stx-stimulation.

5.2. Colonization mechanism of *E. coli* O157:H7

EPEC and EHEC have to adhere to intestinal cells to be pathogenic. Binding to host intestinal epithelial cells leads to a typical A/E lesion, characterized by a loss of microvilli, intimate bacterial attachment to the host cell and actin condensation underneath adherent bacteria. To generate this lesion, A/E bacteria use a conserved type III secretion system (TTSS), a complex structure at the cell surface resembling a molecular syringe. The LEE encodes the structural components of the TTSS, translocated and effector proteins, as well as several gene regulators and chaperones.

Colonization by EPEC and EHEC is a three-step-process: i) initial adhesion, ii) signal transduction and cytoskeletal rearrangements and iii) intimate adhesion and pedestal formation (Donnenberg et al., 1997) (Figure 2). Several molecules have been implicated in the initial binding step, such as OmpA (Torres and Kaper, 2003) and long polar fimbriae (Torres et al., 2002). However, their exact role remains unclear. It is certain that initial binding of EHEC to host cells is dependent on EspA-filaments (Ebel et al., 1998), extending from the bacterial surface to make contact with host cells (Knutton et al., 1998). EspB and EspD form a pore in the host cell membrane, allowing the translocation of effector molecules through the hollow needle-like structure formed by EspA-molecules (Kresse et al., 1999; Daniell et al., 2001). The translocated intimin receptor (Tir) is inserted into the host cell membrane, and interacts with the bacterial outer membrane protein intimin, thereby establishing an intimate adhesion with the host cell (DeVinney et al., 1999). Intimin is also known to bind the host cell receptors β1 integrins and nucleolin (Frankel et al., 1996; Sinclair and O'Brien, 2002) but the role of these interactions *in vivo* is not clear. Upon intimate adhesion, bacteria multiply and develop microcolonies on the epithelial cell surface (Tatsuno et al., 2000).

Tir adopts a hairpin-like structure in the host cell membrane, where the extracellular domain interacts with intimin, and the N- and C-termini are located in the cytosol, interacting with host cell proteins. In contrast to EPEC Tir (Rosenshine et al., 1996), EHEC Tir does not have to be phosphorylated to initiate the cascade of events leading to the formation of an A/E lesion (DeVinney et al., 1999). Instead, Tir is linked indirectly to the actin cytoskeleton by the Tir-cytoskeleton coupling protein TccP (also named $EspF_U$) (Garmendia et al., 2004), via a host protein, the insulin receptor tyrosine kinase substrate (IRTKS), a homologue of the insulin receptor substrate protein of 53 kDa (IRSp53) (Vingadassalom et al., 2009; Weiss et al., 2009). IRSp53 family members are key regulators of membrane and actin dynamics. TccP interacts with

the neural Wiskott Aldrich syndrome protein (N-WASP) to activate the Arp2/3 complex which mediates actin nucleation. Actin condensation underneath adherent bacteria leads to the formation of a pedestal, on which the bacteria rest (Knutton et al., 1989). Following attachment and effector translocation, expression of the EspA needle complex and intimin is downregulated (Knutton et al., 1997; Knutton et al., 1998; Dahan et al., 2004).

It is clear from this cascade of events that EHEC has evolved an ingenious strategy to attach itself to the host cell, inserting not only its own receptor and a linker molecule but also usurping host regulators of cytoskeleton dynamics. It can be speculated that such a tight interaction between bacterium and host cell will impair the removal of EHEC by immune cells. Furthermore, EHEC has developed several strategies to evade the host immune system, which will be further discussed below.

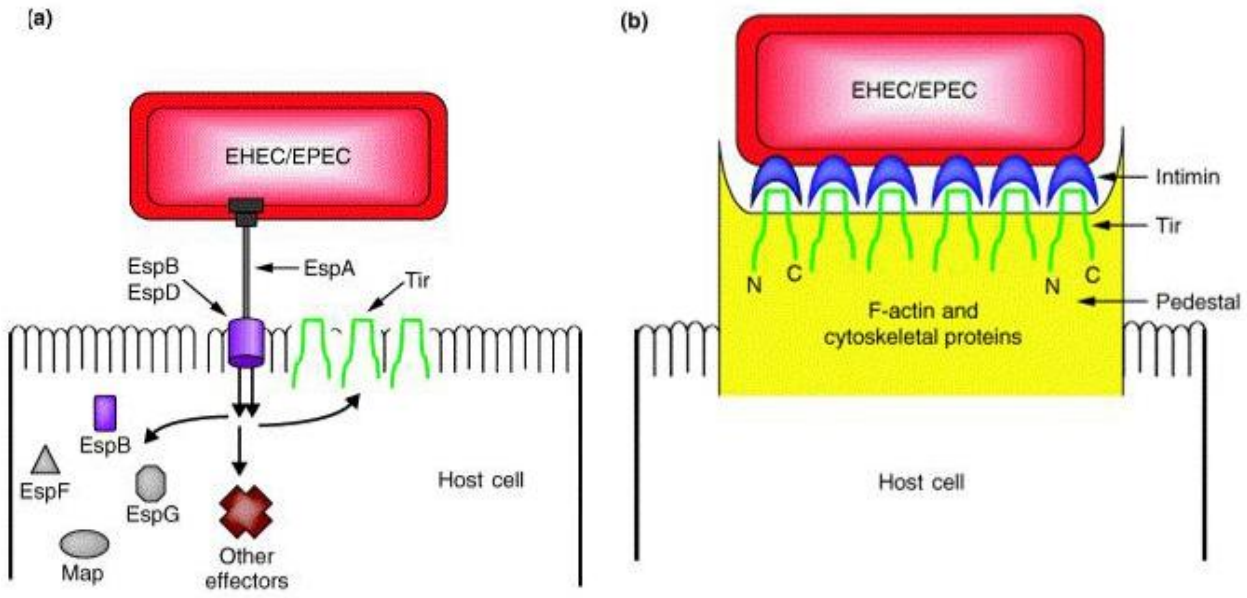


Figure 2: Model of translocation of bacterial effectors into host cells. (a) Upon establishment of initial contact by a mostly unknown mechanism, EPEC and EHEC use a type III translocation apparatus to inject bacterial effector proteins into mammalian cells. These bacteria translocate a number of proteins: EspB and EspD, which form a translocon in the plasma membrane; the cytoplasmic proteins EspF, EspG and Map (there is also a cytoplasmic pool of EspB); the translocated intimin receptor Tir, which inserts into the plasma membrane; and other effectors. (b) Membrane-localized Tir contains a central extracellular domain that binds to the bacterial outer membrane protein intimin, and amino- and carboxy-terminal cytoplasmic domains that interact with cytoskeletal elements. The interaction between Tir and intimin is the final bacterial signal to trigger the assembly of actin into pedestals within host cells. Adapted from Campellone and Leong (2003).

5.3. Plasmid pO157

All EHEC strains possess a large plasmid of 90 kb or 60MDa, the pO157 plasmid. Plasmids of different *E. coli* O157:H7 strains are highly conserved, differing only at the single nucleotide polymorphism level (Zhang et al., 2006). The complete sequence of the pO157 plasmid was determined, revealing several putative virulence factors (Burland et al., 1998; Makino et al., 1998). An overview of the most prominent virulence factors encoded on the pO157 is given here.

Enterohaemolysin or EHEC-haemolysin is related to alpha-haemolysin but is not identical (Schmidt et al., 1995). The *hlyCABD* operon contains four genes, encoding a pore-forming cytolysin and its secretion apparatus. Toxicity results from the insertion of enterohaemolysin into the cytoplasmic membrane of the target mammalian cells disrupting permeability control. How enterohaemolysin contributes to the pathogenesis is not well understood. It is possible that the haemoglobin released by the action of the haemolysin provides a source of iron, thereby stimulating the growth of EHEC bacteria in the intestine (Law and Kelly, 1995). In addition to lysing erythrocytes, the toxin lyses bovine but not human lymphocytes (Bauer and Welch, 1996). Enterohaemolysin is expressed during *in vivo* infection since it was found to reac with sera from HUS patients (Schmidt et al., 1995) and bovine colostra (Lissner et al., 1996).

KatP is a catalase-peroxidase encoded on the O157 plasmid, which protects EHEC from oxidative stress (Brunder et al., 1996; Uhlich, 2009). The occurrence of the *katP* gene in EHEC strains is associated with the *hly* operon (Brunder et al., 1997).

EspP is a secreted serine protease that cleaves pepsin A and human coagulation factor V (Brunder et al., 1997). EspP contributes to adherence of *E. coli* O157:H7 to primary bovine rectal cells and to colonization in vivo as an espP deletion mutant was excreted in lower numbers by calves than the parent strain (Dziva et al., 2007a).

StcE or secreted protease of C1 esterase inhibitor (C1-INH) is a metalloprotease that specifically cleaves C1-INH, a host regulator of multiple proteolytic cascades, including the classical and alternative complement pathway, coagulation and contact activation pathways (Caliezi et al., 2000, Jiang et al., 2001). Resulting pathology from *E. coli* O157:H7 infections may include localised proinflammatory and coagulation responses, causing tissue damage, intestinal oedema and thrombotic abnormalities (Lathem et al., 2002). StcE is thought to promote adherence through destruction of proteins in the glycocalyx thereby allowing O157:H7 to come

into close contact with the intestinal mucosa (Grys et al., 2005) and to aid *E. coli* O157:H7 in resisting the complement pathway of the innate immune response (Lathem et al., 2004).

Lymphostatin/Efa-1/ToxB are homologous proteins in respectively EPEC, non-O157 EHEC and *E. coli* O157:H7. Lymphostatin inhibits lymphocyte proliferation of human peripheral blood lymphocytes (PBMC) and the mitogen-activated synthesis of proinflammatory cytokines (Klapproth et al., 1995; Klapproth et al., 2000), as well as human and murine intestinal lymphocytes (Klapproth et al., 1996; Malstrom and James, 1998) but has no effect on the formation of A/E lesions. The *efa-1* gene (EHEC factor for adherence) is 99.9% identical in nucleotide sequence to the EPEC *lifA*, mediates bacterial adherence to cultured epithelial cells on its own and influences intestinal colonization of calves by non-O157 EHEC strains (Nicholls et al., 2000; Stevens et al., 2002b). *E. coli* O157:H7 strains encode a truncated *efa-1'* gene, that influences bacterial adhesion, but also contain a functional homologue protein to lymphostatin, namely *toxB* (Klapproth et al., 2000). The ToxB protein shares a considerable homology (28% of identical amino acids and 47% of similar amino acids) with the product of *efa-1/lifA* (Morabito et al., 2003). Tatsuno and colleagues showed that the product of the *toxB* gene contributes to the adherence of EHEC O157 to Caco-2 cells through the promotion of the production and/or the secretion of type III secreted proteins (Tatsuno et al., 2001). A plasmid-cured *E. coli* O157 strain could still adhere to Caco-2 cells, but developed fewer microcolonies than the parent strain. Furthermore, the plasmid-cured strain could not inhibit IL-2 and IL-4 synthesis in mitogen-activated PBMC (Klapproth et al., 2000). However, it is unclear whether this effect can be solely attributed to ToxB as other cytotoxins encoded on the plasmid such as enterohaemolysin or StcE may also be responsible. In contrast to non-O157 strains, mutation of *E. coli* O157 ToxB did not affect intestinal colonization of calves and sheep (Stevens et al., 2004).

Lymphostatin has recently been shown to contribute to pathogenesis of *C. rodentium* by disrupting the intestinal barrier via the modulation of Rho GTPases (Babbin et al., 2009). It is unclear how one protein can exhibit such different functions as a role in adhesion and modulation of the host immune response. Together, these data indicate that lymphostatin and its homologues are important virulence factors in A/E bacteria.

Karch et al. (1987) first reported that the O157 plasmid is associated with the expression of fimbriae that enhance bacterial adherence to epithelial cells. However, other studies have shown that the plasmid has no effect on adhesion or that it resulted in reduced or even increased adherence (Tzipori et al., 1987; Toth et al., 1990; Fratamico et al., 1993; Nataro and

Kaper, 1998). These conflicting results may be explained by the use of animal models such as mice, gnotobiotic piglets and rabbits which do not correlate well with human disease caused by *E. coli* O157:H7. It has recently been reported that pO157 influences colonization of the bovine terminal rectum. Strains missing the plasmid were cleared faster than the wild type strain, showing its requirement for long-term colonization in cattle (Sheng et al., 2006b; Lim et al., 2007b). Enterohaemolysin deletion mutants did not influence persistence (Lim et al., 2007b).

5.4. Effector molecules

Although EHEC remains extracellular, it has extensive intracellular effects. Host cell signalling pathways are subverted and manipulated, resulting in efficient colonization and evasion of host immune responses. These effects are the result of the coordinated action of a large repertoire of effector molecules that are injected into the cell by the TTSS (Figure 3). Many of these translocator effectors have multiple or redundant functions. The first effectors that were discovered were all encoded on the LEE, but more recently, effectors encoded outside the LEE have been found in all A/E pathogens. These non-LEE effector molecules are carried on prophages or other pathogenicity islands. The role of the effector proteins has mostly been investigated in EPEC.

In 2009, 21 LEE and non-LEE effectors were known in EPEC E2348/69, the prototype strain used worldwide to study EPEC infection (Iguchi et al., 2009). The EHEC O157 Sakai strain encodes 49 potentially functional effectors, representing the most complex repertoire among A/E pathogens (Tobe et al., 2006). The roles of other recently identified effectors such as EspK, EspM and EspT have not yet been clearly defined (Tobe et al., 2006; Vlisidou et al., 2006b; Arbeloa et al., 2009; Bulgin et al., 2009). The most important findings regarding effector molecules are listed below.

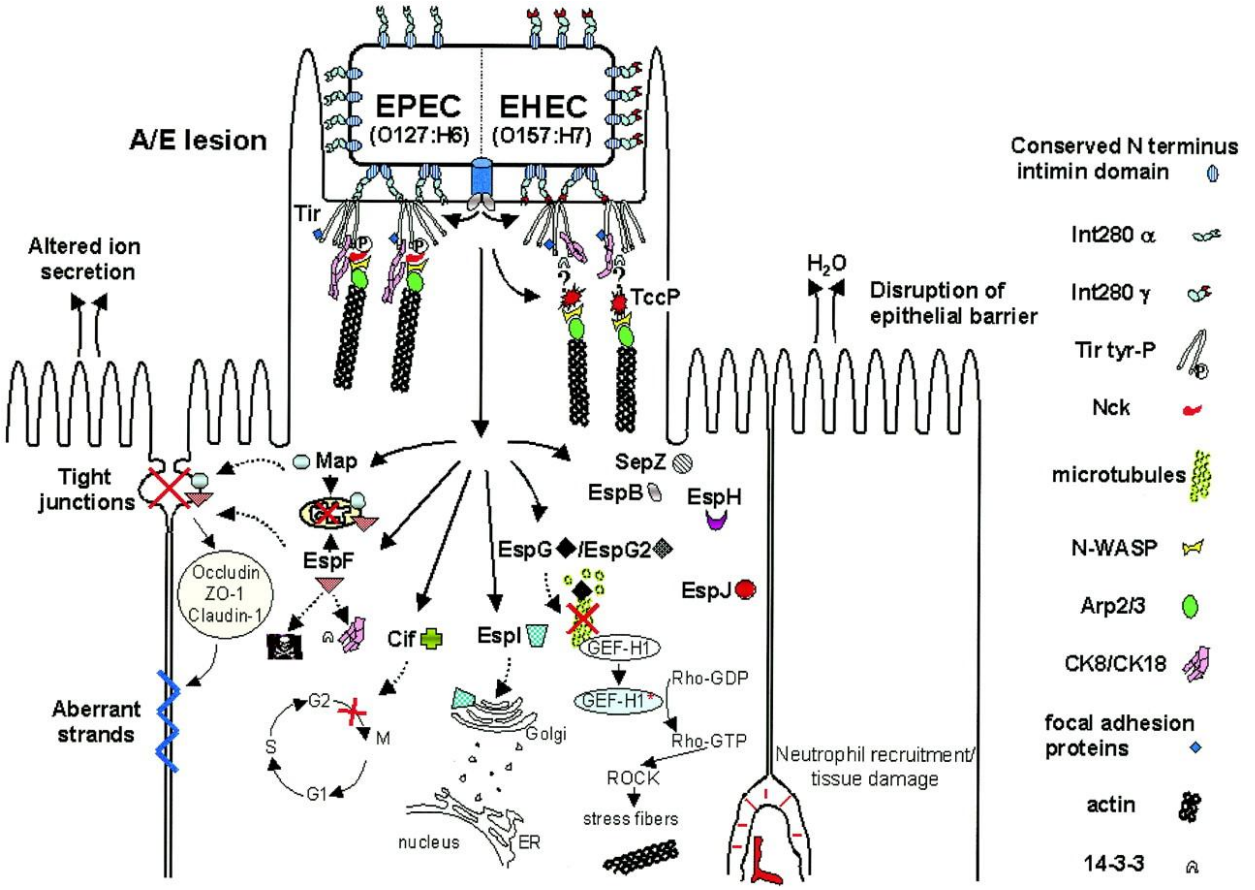


Figure 3: EPEC and EHEC translocate LEE-encoded and other effector proteins into the host cell cytosol. These effectors trigger cytoskeleton rearrangements (Tir, EspH, EspF, and EspG), disruption of the epithelial barrier (EspF and Map), cytotoxicity (EspF and Cif), and host cell responses that ultimately generate watery diarrhoea. Different from EPEC Tir, EHEC Tir is not tyrosine phosphorylated and does not interact with Nck in order to trigger actin polymerization at the site of bacterial adhesion; instead, EHEC translocates an effector protein, TccP/EspFu, which has an Nck-like activity and is essential to promote the reorganization of the actin cytoskeleton underneath adherent EHEC bacteria. ER, endoplasmic reticulum. (Garmendia et al., 2005).

5.4.1. LEE-encoded effectors

Mitochondrion-associated protein (Map) was the second effector protein that was identified after Tir. Map displays three distinct and independent functions: (i) in initial stages of EPEC/EHEC infection, Map is responsible for the transient formation of filopodia at the site of bacterial infection, a process that is dependent on the small regulatory GTPase protein Cdc42, which normally directs filopodia formation within host cells (Kenny et al., 2002); (ii) it interferes with the cellular ability to maintain mitochondrial membrane potential, triggering the formation of misshapen mitochondria, mitochondrial swelling and damage (Kenny and Jepson, 2000); and (iii) it is essential for the disruption of intestinal barrier function and alteration of tight junctions, and this activity is independent of the mitochondrial targeting (Dean and Kenny, 2004). Interestingly, Map signalling is inhibitory to Tir-intimin-triggered pedestal formation (Kenny et al., 2002).

EspB forms a pore in the host cell membrane, through which effector proteins are translocated. In addition, it is translocated itself to the cytosol near the site of bacterial attachment (Taylor et al., 1998) and is thought to have an effector function with a role in cytoskeleton modulation. Cells transfected with EPEC EspB display altered morphology with a reduced number of stress fibres (Taylor et al., 1999). Kodama and co-workers identified the cytoskeleton-associated protein α-catenin as the direct binding partner of *E. coli* O157:H7 EspB, but its role in actin accumulation and A/E lesion formation is unclear (Kodama et al., 2002). EPEC is known to inhibit phagocytosis (Goosney et al., 1999), mediated by EspB as shown recently (Iizumi et al., 2007). When injected into the host cell, EspB inhibits the interaction between actin and myosins, a superfamily of proteins that interact with actin filaments and mediate essential cellular processes, including microvillus formation and phagocytosis (Iizumi et al., 2007).

EspF from EPEC plays a central role in decreasing transepithelial electrical resistance (TER) and in altering the intestinal epithelial tight junction structure *in vitro* (McNamara et al., 2001). The ability of EspF to alter the TER, but not the delivery into host cells, was found to be dependent on expression of intimin, showing that EPEC can control the activity of effector molecules independent of Tir (Dean and Kenny, 2004). Although originally EspF was thought not to be involved in A/E lesion formation, it has been shown recently that EspF has an affinity for profilin, Arp2/3 and actin (Peralta-Ramirez et al., 2008). Furthermore, EPEC EspF can inhibit phagocytosis (Quitard et al., 2006) and it was shown that EspF disrupts nucleolin by a process regulated by mitochondrial dysfunction, possibly targeting the cell's energy resources (Dean et al., 2010).

However, there are marked differences between EPEC and EHEC. EHEC-induced reduction in TER is significantly slower and more modest in comparison with that induced by EPEC (Viswanathan et al., 2004). Furthermore, deletion of the *espF* gene promoted the accumulation of polymorphonuclear cells in the rabbit intestinal mucosa, which suggests that EspF may be important to dampen the host inflammatory response to EHEC (Ritchie and Waldor, 2005).

EspG and its homologue EspG2 bind to tubulins and cause localized microtubule depolymerisation, resulting in actin stress fibre formation (Matsuzawa et al., 2004; Hardwidge et al., 2005). They are implicated in the diarrhoeal progress of disease caused by *C. rodentium* infections, by partially altering the location of aquaporin water channels (Guttman et al., 2007). Nevertheless, an *espG* mutant strain displayed only slight attenuation in animals in the rabbit

EPEC infection model and the *C. rodentium* mouse model (Elliott et al., 2001; Deng et al., 2004; Mundy et al., 2004). However, in a rabbit EHEC infection model, an *espG* deletion mutant formed A/E lesions in the colon but ileal colonisation was reduced approximately 10-fold in comparison with the wild type strain (Ritchie and Waldor, 2005).

EspH is a modulator of the host cytoskeleton, by down-regulating filopodium formation and promoting pedestal formation in HeLa cells infected with EPEC or EHEC (Tu et al., 2003). It is thought to promote EHEC colonization in the rabbit intestinal tract (Ritchie and Waldor, 2005).

SepZ (also named EspZ) is hypervariable among A/E pathogens, with sequences sharing between 60 and 81% amino acid identity between EHEC and EPEC SepZ (Kanack et al., 2005). Interestingly, the allele subtype of the *espZ* gene correlates with the intimin subtype among EHEC strains (Gilmour et al., 2006). A specific 8 to 10 amino acid loop between 2 predicted transmembrane domains is the most hypervariable part of the protein, suggesting that it may represent a region that interacts directly with host proteins (Kanack et al., 2005). Although it is known that SepZ is translocated early in the infection process and at similar levels as the important effector Tir (Mills et al., 2008), a role in pathogenesis of A/E pathogens has not yet been defined (Deng et al., 2004; Kanack et al., 2005). Recently, it has shown that EspZ/SepZ interacts with CD98 and protects the host cell against EPEC-mediated cytotoxicity (Shames et al., 2010).

5.4.2. Non-LEE effectors

Cycle inhibiting factor (Cif) of EPEC and EHEC strains triggers an irreversible cytopathic effect in epithelial cells, characterized by progressive recruitment of focal adhesions, assembly of stress fibres and the arrest of the cell proliferation in G1 and G2 phases (Marches et al., 2003; Samba-Louaka et al., 2008). Cif not only induces cell cycle arrest but eventually provokes delayed cell death (Samba-Louaka et al., 2009). Cell cycle inhibitors might impair epithelial-barrier integrity allowing the entry of pathogenic bacteria into the body or prolonging their existence by blocking the shedding of epithelia (Marches et al., 2003). The latter study suggests that targeting of intestinal T-cells by Cif could have serious effects on the innate and adaptive immune responses. Unfortunately, this has not been investigated yet, and the role of Cif in EPEC and EHEC pathogenesis *in vivo* remains to be elucidated. Cif was detected in different EHEC serotypes but not in *E. coli* O157:H7 strains (Marches et al., 2003; Loukiadis et al., 2008).

Moreover, Loukiadis et al. showed that not all *cif*-positive strains encode a full-length gene or a functional protein.

EspJ of EPEC and EHEC inhibits opsono-phagocytosis by macrophages, possibly through targeting of a host cell molecule normally involved downstream of the phagocytic receptors FcγR and CR3 as EspJ inhibits phagocytosis mediated by these receptors (Marches et al., 2008).

Several non-LEE encoded effectors (Nle), NleA to I, have been described. Most of them were originally identified in the genome of *C. rodentium* and are conserved among A/E pathogens (Deng et al., 2004; Garcia-Angulo et al., 2008). The functions of some of these proteins have been investigated, mostly in EPEC. **NleA** is implicated in disrupting intestinal tight junctions between epithelial cells by EPEC, and this effect may be mediated by disruption of cellular protein trafficking (Kim et al., 2007; Thanabalasuriar et al., 2010). Results from EHEC infection of HeLa cells suggest that NleA may contribute to resisting host clearance of infection (Gruenheid et al., 2004). **NleB** and **NleE** are involved in subverting innate immune responses to EPEC, whereby NleE blocks NF-κB p65 nuclear translocation, leading to reduced IL-8 production (Newton et al., 2010) and NleB enhances the inhibition of NF-κB by the NleE protein (Nadler et al., 2010). **NleD** was found to play a role in the intestinal colonization of calves using signature-tagged mutagenesis (Dziva et al., 2004), however this was in contrast with a later study which showed that both **NleC** and **NleD** were not required for colonization of calves and lambs (Marches et al., 2005). **NleF** plays a role in the colonization of gnotobiotic piglets by EHEC (Echtenkamp et al., 2008). **NleG** homologues actually represent a large family of effector proteins found in pathogenic *E. coli*, with fourteen members in *E. coli* O157:H7 alone. NleG proteins exhibit a strong autoubiquitination activity *in vitro* but their role *in vivo* has not been investigated yet (Wu et al., 2010). **NleH** was shown to cause an increase in NF-κB activity and TNF-α expression in the mouse colonic mucosa (Hemrajani et al., 2008), and modulates apoptotic responses during EPEC and *C. rodentium* infections by inhibiting caspase activation (Hemrajani et al., 2010). In EHEC, NleH binds to the human ribosomal protein S3, which is a subunit of NF-κB, and thereby contributes to the disruption of host immune responses (Gao et al., 2009). **NleI** was identified in EPEC and facilitates secretion of other effector molecules, including NleA, F and H (Li et al., 2006).

In summary, EPEC and EHEC use multiple proteins with redundant functions to subvert host cell functions. EPEC and EHEC are related pathogens and share many virulence

characteristics. However, marked differences exist and it would be unwise to attribute functions of effectors identified in EPEC without prior investigation to the EHEC effector homologue.

5.5. Effect of *E. coli* O157:H7 on the host cell

From the still growing list of effector molecules and virulence factors, it is clear that *E. coli* O157:H7 exerts many effects on the host cell. Infection with *E. coli* O157:H7 triggers a complex interplay of communication between the bacteria and the host cell. Activated enterocytes produce cytokines, which attract leucocytes to the site of infection and induce mucosal inflammation. Host cells recognize certain structures on the bacteria, the pathogen-associated molecular patterns (PAMPs). Several molecules of *E. coli* O157:H7 have been implicated in this process. OmpA of *E. coli* O157:H7 induced mouse dendritic cells to secrete IL-1, IL-10, and IL-12 in a dose-dependent manner, and this effect was enhanced by incubation of the bacteria with OmpA-specific antibodies, forming immune complexes (Torres et al., 2006). Stx and H7 flagellin are other potent inducers of cytokine secretion (Thorpe et al., 1999; Rogers et al., 2003; Miyamoto et al., 2006; Gobert et al., 2008), as well as proteins of the LEE, but intimate adhesion is not a prerequisite for secretion of IL-8 and CCL20 (Gobert et al., 2008). An as yet unidentified molecule of *E. coli* O157:H7 inhibits IFN-γ-induced activation of the JAK/STAT pathway upon adhesion, and this molecule is not a LEE or TTSS protein, Stx1 or Stx2, and is not encoded on the pO157 plasmid (Ceponis et al., 2003; Jandu et al., 2007). Not only IFN-γ-induced signalization is impaired*, E. coli* O157:H7 inhibits IFN-γ secretion from lymphocytes (Klapproth et al., 2000). IFN-γ might play an important role during EHEC infection, as suggested by the inability of IFN-γ-knockout mice to clear *C. rodentium* infection (Simmons et al., 2002). Furthermore, *E. coli* O157:H7 interferes with NF-κB activation initiated by TNF-α, suppressing IL-1, IL-6 and IL-8 secretion (Hauf and Chakraborty, 2003).

The watery diarrhoea seen in the initial stage of human disease is thought to be caused by a change in TER, as seen in infected human intestinal T84 cells. *E. coli* O157:H7 alters the distribution of tight junction-associated proteins, thereby influencing the epithelial permeability (Philpott et al., 1998). T84 cells have been used to study another aspect of the pathogenesis, namely the effect of Stx on human intestinal epithelial cells. The latter do not express the Stx-receptor (Schuller et al., 2007), but are able to translocate Stx into the blood stream (Thorpe et al., 1999). It was shown that *E. coli* O157:H7 actually suppresses the inflammatory host response to cytokines and Stx, by inhibiting the Stx-induced IL-8 secretion. It seems that *E. coli* O157:H7 is

able to protect the integrity of the epithelium by dampening the host response to its toxin (Bellmeyer et al., 2009).

Immunomodulation by Stx has also been implicated in the ability of *E. coli* O157:H7 to persist in cattle. Stx1 can block the proliferation of bovine lymphocytes *in vitro* (Menge et al., 1999). More specifically, bovine CD8+ and CD21+ cells are involved, and to a lesser extent CD4+ cells. This effect is not mediated by cytokines but is the result of the direct action of Stx on bovine lymphocytes (Menge et al., 2003). Bovine B- and T-lymphocytes, including ileal and colonic intraepithelial lymphocytes, express Gb3/CD77 molecules *in vitro* that function as Stx-receptors (Menge et al., 2001; Stamm et al., 2002; Menge et al., 2003). In addition, it has been shown that another *E. coli* O157:H7 molecule, lymphostatin, has a similar blocking effect on bovine lymphocytes (Abu-Median et al., 2006).

6. Immune response to *E. coli* O157:H7

Humans and animals respond immunologically to *E. coli* O157:H7 infections. In contrast to what has since long been perceived, *E. coli* O157:H7 is not a commensal of the bovine host, but induces inflammation and can be considered as a bovine pathogen. Although cattle do not experience clinical symptoms, it has been discovered recently that *E. coli* O157:H7 induces small mucosal haemorrhages and focal petechiae in the intestine of colonized cattle, that remain visible several months after ceasing of shedding (Baines et al., 2008).

6.1. Human response

Serum antibodies reactive to *E. coli* O157:H7 lipopolysaccharide (LPS) have been repeatedly reported in haemorrhagic colitis and HUS-patients (Bitzan et al., 1991; Flores et al., 1997; Jenkins and Chart, 1999; Tanaka et al., 2000) and can be used for serodiagnosis of *E. coli* O157:H7 infections that otherwise would be unnoticed due to concurrent infections (Flores et al., 1997) or when faecal bacteria cannot be detected. IgM and IgA antibodies to O157 LPS were also detected in breast milk of an *E. coli* O157-infected pregnant woman (Tanaka et al., 2000) and in saliva of children with HUS (Ludwig et al., 2002b). However, serum antibodies in HUS-patients were not long-lasting and disappeared 5 (IgA) or 11 weeks (IgG and IgM) after the onset of diarrhoea (Ludwig et al., 2002a). Recurrent *E. coli* O157:H7 infections with HUS have been reported (Siegler et al., 1993), indicating that O157 LPS-specific antibodies may not protect against subsequent *E. coli* O157:H7 infections.

Besides LPS, patients react against intimin, EspA, EspB and Tir (Jenkins et al., 2000; Li et al., 2000). Titres against these proteins were strongly elevated by day 8 after hospitalisation due to *E. coli* O157:H7 infection, in comparison with titres of acute-phase sera (Li et al., 2000). The response decreased by day 60 and was highest against Tir. However, Karpman et al. (2002) observed the highest responses against EspB during the acute phase. Reactive antibodies are sometimes present in sera from healthy control subjects, indicating the similarity of EHEC antigens between different *E. coli* serotypes (Jenkins et al., 2000; Karpman et al., 2002).

Serum antibodies to enterohaemolysin and H7 flagella have been detected, but are not useful for serodiagnosis of *E. coli* O157:H7 infections as they were only present in a minority of patients (Jenkins and Chart, 1999). On the other hand, patients with HUS did not have serum antibodies to either A or B subunits of Stx1 or Stx2 (Chart et al., 1993).

Studies of cytokine responses focus on HUS-patients. Typically, diarrhoea precedes the development of HUS by several days. Westerholt et al. (2000) concluded from a study conducted in children with diarrhoea caused by EHEC that high IL-8 and low IL-10 levels are risk factors for developing HUS. In contrast, Murata et al. (1998) found increased levels of IL-10 in EHEC-related HUS, as well as increased TNF-α, IL-6, IL-8 and granulocyte colony-stimulating factor. The host immune response differs between uncomplicated *E. coli* O157:H7 infection and HUS-development, and is likely a reflection of inter-person variability rather than a O157-specific immune response (Proulx et al., 1998).

The secreted cytokines attract leucocytes to the site of infection. Histological analysis of colonic biopsies of *E. coli* O157:H7-infected patients showed infiltration of monocytes and neutrophils in the crypts and the lamina propria (Griffin et al., 1990; Shigeno et al., 2002).

6.2. Response in ruminants

Naturally infected cattle develop serum antibody responses against various *E. coli* O157:H7 antigens such as Stx and LPS (Pirro et al., 1995; Cristancho et al., 2008). Bovine colostrum contains antibodies directed against Stx, LPS and *E. coli* O157:H7 proteins involved in adhesion (Lissner et al., 1996; Widiasih et al., 2004b; Vilte et al., 2008), which could explain the low prevalence in pre-weaned calves (Garber et al., 1995).

Whereas anti-Stx1 antibodies were detected in all colostrum and serum samples obtained from 24 dams immediately after parturition, they were present only in a minority of milk samples and in low amounts. The anti-Stx1 antibodies were transferred to newborn calves

via the colostrum, but titres declined by six weeks of age whereafter calves became increasingly susceptible to natural *E. coli* O157:H7 infection (Frohlich et al., 2009). Anti-Stx2 antibodies were only rarely detected under natural conditions (Frohlich et al., 2009) or after repeated experimental infections (Johnson et al., 1996; Hoffman et al., 2006).

Although detection of serum antibodies in humans can be used to diagnose *E. coli* O157:H7 infection, the presence of serum antibodies is probably not a useful parameter to determine the infection status of ruminants. Seroconversion to active IgM and IgG anti-LPS antibody responses in some calves was not associated with isolation of the bacteria (Cristancho et al., 2008), indicating possible cross-reactivity to LPS of non-O157 strains or other bacterial species. However, limited cross-reactivity to TTSS proteins of O157 and non-O157 strains was reported (Asper et al., 2007). Recently it has been discovered that the subtilase cytotoxin of *E. coli* O157:H7 blocks secretion of newly synthesized antibodies *in vitro*, but this effect remains to be investigated *in vivo* during the course of *E. coli* O157:H7 infection (Hu et al., 2009).

Antibodies to a greater variety of *E. coli* O157:H7 antigens have been shown in experimentally infected cattle, including Stx-, LPS-, and TTSS protein-specific antibodies. These antibodies inhibit *E. coli* O157:H7 adhesion to cells *in vitro* (Paton et al., 1998), but pre-existing antibody titres do not prevent colonization of cattle following experimental inoculation (Bretschneider et al., 2007b). However, it has been speculated that acquired immunity may account for reduced shedding after repeated inoculations (Johnson et al., 1996; Sanderson et al., 1999; Naylor et al., 2007). Still, the immune response during the course of an *E. coli* O157:H7 remains largely unknown.

Mucosal responses following experimental infection of cattle include infiltration of granulocytes in the lamina propria of the terminal rectum, and rectal IgA antibodies recognizing several O157 antigens (Bretschneider et al., 2008; Nart et al., 2008a; Nart et al., 2008b). Faecal IgG and IgA antibodies were not detected (Bretschneider et al., 2007b).

Results from *in vitro* studies have generated the hypothesis that *E. coli* O157:H7 can actively suppress the host immune response (Menge et al., 1999), and this was supported by the reduced cellular responses of cattle experimentally infected with an Stx2-positive strain compared to those infected with an Stx2-negative strain (Hoffman et al., 2006).

Anti-H7 flagellin antibodies purified from immunized calves, were capable of partially blocking adhesion of *E. coli* O157:H7 to primary rectal epithelial cells (McNeilly et al., 2008). Interestingly, these IgG and IgA antibodies recognised different epitopes and the IgG antibodies

interfered with Toll-like receptor (TLR) 5 signalling (McNeilly et al., 2010). Inhibition of TLR signalling may reduce vaccine efficacy by interfering with innate responses to the bacterium, and may explain that although less animals became colonized after vaccination, animals that were colonized sustained bacterial peak levels for longer periods than did the unvaccinated controls (McNeilly et al., 2008). Rectal immunisation induced rectal IgA, which had no effect on bacterial shedding, in contrast to IgG induced by systemic immunization that reduced shedding and delayed the peak of shedding (McNeilly et al., 2008).

Immune responses of sheep against *E. coli* O157:H7 have not been studied before and are investigated in this thesis.

Part II: Aims of the study

Aims of the study

Whereas many investigations have focused on the colonization mechanisms of *E. coli* O157:H7, the nature of the immune response against this pathogen has received only limited attention. It has been hypothesized that infection with *E. coli* O157:H7 can persist in ruminants due to suppression of the host immune system. While it is known that cattle develop antibodies after natural and experimental infection with *E. coli* O157:H7, the immune response of sheep against *E. coli* O157:H7 has not been studied before.

The general aim of this thesis was to gain insight in the interaction of *E. coli* O157:H7 with the immune system of sheep.

The following questions were addressed:

Does experimental infection of sheep with *E. coli* O157:H7 lead to persistent colonization?

- By a rectal inoculation route? *(Chapter 1)*
- By an oral inoculation route? *(Chapter 2)*
- Where is the infection localized? *(Chapter 1 & 2)*

Does infection result in an immune response that protects against re-infection?

- What is the systemic immune response? *(Chapter 1, 2 & 3)*
- What is the local immune response? *(Chapter 1, 2 & 3)*

What is the role of the immune response in clearance of *E. coli* O157:H7 infection?

- What is the influence of preceding immunisation? *(Chapter 3)*

Part III: Experimental work

Chapter 1:

Rectal inoculation of sheep with *E. coli* O157:H7 results in persistent infection in the absence of a protective immune response

Based on: Vande Walle K., Atef Yekta M., Verdonck F., De Zutter L., Cox E., 2010. Rectal inoculation of sheep with *E. coli* O157:H7 results in persistent infection in the absence of a protective immune response. Veterinary Microbiology, in press, doi: 10.1016/j.vetmic.2010.06.033.

1.1. Abstract

Escherichia coli (E. coli) O157:H7 can cause haemorrhagic colitis and the haemolytic uremic syndrome in humans. Ruminants are the main reservoir for this bacterium: they can harbour the bacteria in the gastro-intestinal tract without showing clinical symptoms. The reason for this persistence is still unclear, although it has been suggested that *E. coli* O157:H7 can suppress the immune system. To investigate the effects on the immune system of ruminants, an infection model is needed that mimics a long-term infection as it can occur in both sheep and cattle. As the terminal rectum has recently been identified as a primary colonization site in cattle, we developed a rectal inoculation model for sheep and used this model to study immune responses against selected virulence factors of *E. coli* O157:H7 (intimin, EspA and EspB). Sheep were infected and re-infected when *E. coli* O157:H7 excretion was no longer detectable. The animals did not develop serum or local antibody responses but showed a cellular response against EspA and intimin respectively 9 en 16 days after infection. This response was also present 5 days after re-infection, albeit lower, and did not prevent animals from being re-infected. These results demonstrate that *E. coli* O157:H7 can be persistently present in the large intestine of sheep without inducing a clear protective immune response.

1.2. Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is associated with haemorrhagic colitis and the haemolytic uremic syndrome in humans (Paton and Paton, 1998), with food and drinking water contaminated with animal faeces being the most important sources of infection for humans (Hancock et al., 2001). Ruminants transiently harbour *E. coli* O157:H7 in their gastrointestinal tract, and more specifically the terminal rectum has been identified as a primary colonization site of *E. coli* O157:H7 in cattle (Naylor et al., 2003). Given the reported variation in duration of faecal excretion after oral inoculation (Brown et al., 1997; Cookson et al., 2002; Grauke et al., 2002; Sheng et al., 2004) and the need for an experimental model mimicking a more long-term infection in cattle, Sheng and co-workers (2004) used rectal swab inoculation to target the primary colonisation site of *E. coli* O157:H7. This resulted in consistent long-term colonization of adult steers, with excretion lasting more than one month post inoculation.

Cattle develop serum antibodies against virulence factors of *E. coli* O157:H7 (such as intimin, EspA, EspB, Tir, LPS and Shiga toxin) after natural and experimental infection (Pirro et al., 1995; Johnson et al., 1996; Hoffman et al., 2006; Bretschneider et al., 2007b), but the role of the

immune system in persistence of *E. coli* O157:H7 is poorly understood. It has been suggested that protective immune responses occur, based on reduced shedding following immunization with type III secreted proteins (Potter et al., 2004; Smith et al., 2009b) and after repeated experimental infections (Sanderson et al., 1999; Naylor et al., 2007). A better knowledge of the immune response that occurs during infection may lead to improved understanding of the mechanisms behind persistence and clearance of the infection. This knowledge might be crucial to create effective control measures of EHEC colonization in ruminants, thereby reducing the spread to humans.

Most studies focus on cattle, but sheep are an established ruminant model for *E. coli* O157:H7 colonization after oral inoculation (Wales et al., 2001a; Wales et al., 2001b; Cookson et al., 2002; Woodward et al., 2003; Best et al., 2009). In the present study, rectal inoculation of sheep was used to develop a persistent infection model, and to study the immune response to virulence factors of *E. coli* O157:H7.

1.3. Material and methods

1.3.1. Bacterial inoculums

NCTC12900 is a well characterized Shiga toxin-negative *E. coli* O157:H7 strain with spontaneous nalidixic acid resistance (Dibb-Fuller et al., 2001; Wales et al., 2002; Woodward et al., 2003). For preparation of bacterial inocula, NCTC12900 was grown overnight in Luria Bertani broth at 37°C while shaking (200 rpm), washed and resuspended in PBS to the desired concentration as described below.

1.3.2. Experimental animals

Conventionally reared sheep (Belgian cross-breed, Zootechnical Centre, Leuven, Belgium) were screened at the age of ten weeks, to verify the absence of *E. coli* O157:H7 in faeces and serum antibodies against intimin, EspA and EspB. Selected animals were allowed to acclimatize for one week after arrival in our facilities. Sheep were housed in groups of two to five animals per pen and received a complete pelleted diet and water *ad libitum*. All experimental and animal management procedures were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

1.3.3. Experimental procedures

Experiment 1

A first experiment was performed to test different rectal inoculation methods. Twelve lambs were divided into 5 groups: groups 1 (n=3) and 2 (n=3) were administered a liquid inoculum into the rectal cavity by means of a syringe and a flexible plastic tube, in a low (10^7 CFU, group 1) or high dose (10^{10} CFU, group 2) in a total volume of 20 ml. Groups 3 (n=2) and 4 (n=2) received 10^{10} CFU in 2 ml absorbed in a surgical sponge (Spongostan® Standard) on 1 (group 3) or 2 consecutive days (group 4). Inoculation with a sponge is hereafter referred to as the 1-day-sponge or 2-days-sponge method. Previously, it was determined that at least 10^6 CFU could be released from the sponge after applying light pressure. After each administration of bacteria the tail was held down for at least 10 min to prevent defecation and removal of the inoculum. Group 5 (n=2) did not receive the primary inoculum, but was co-penned with culture-positive sheep 30 days post primary inoculation (dppi) to serve as naturally exposed age-matched controls. On 98 dppi, the animals from groups 3, 4 and 5 were re-infected by the 2-days-sponge method. The experimental design is presented in Figure 1.

Experiment 2

A second experiment was performed to determine the localization of *E. coli* O157:H7 in the gastrointestinal tract after rectal inoculation with the 2-days-sponge method, and to analyse local and cellular immune responses. Thirteen sheep were divided into 3 groups: a re-infected group (n=5), a previously unexposed group (n=3) and a control group (n=5). Animals in the re-infected group received a primary and secondary inoculation on days 0/1 and days 41/42 respectively, whereas animals in the previously unexposed group were only inoculated on days 41/42. Control animals were not inoculated. The experimental design is presented Figure 1. One control and one infected animal (re-infected group) were euthanized on 8 dppi and again on 15 dppi, by captive bolt gun stunning, followed by exsanguination. Intestinal contents were sampled from rectum, colon, caecum, ileum and jejunum, for bacterial enumeration and determination of local antibody responses.

Faecal excretion of *E. coli* O157:H7 and serum antibody responses were assessed as in experiment 1. Additionally, each time when faeces was taken for bacterial enumeration, 1 g faeces was processed for determination of faecal antibodies as described below. Furthermore, blood was taken on 9, 16 and 41 dppi (just before administration of the secondary inoculation),

and 5 days after the secondary inoculation (46 dppi) to determine lymphocyte proliferative responses.

1.3.4. Monitoring of *E. coli* O157:H7 excretion

Faecal excretion of *E. coli* O157:H7 was monitored by direct plating and immunomagnetic separation (IMS) according to the method described by Tutenel et al. (2003) for isolation of *E. coli* O157:H7 from cattle faeces. The detection limit of *E. coli* O157:H7 in sheep faeces was determined to be 10^2 CFU/g by direct plating and 10^1 CFU/g by IMS (data not shown). Nalidixic acid (15 µg/ml) was included in cefixime-tellurite sorbitol MacConkey agar (NalCT-SMAC) to facilitate recognition of NCTC12900 colonies. Briefly, 10 g faeces was diluted in modified tryptone soy broth supplemented with 20 mg/ml novobiocin and homogenized in a stomacher blender. Ten-fold dilution series were plated onto NalCT-SMAC. The remaining broth was enriched for 6h at 42°C and subjected to IMS with O157 Dynabeads® (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Finally, 100 µl was plated onto NalCT-SMAC. Colonies were confirmed to be *E. coli* O157:H7 by latex agglutination test (Oxoid). Colony counts were $\log_{10}$ transformed for data analysis. If *E. coli* O157:H7 was not detected by direct plating but enrichment culture was positive by IMS, the lowest detectable concentration was assigned (10^1 CFU/g). Animals were considered negative after 2 successive negative IMS results.

1.3.5. Recombinant His-tagged virulence factors

Plasmids pCVD468 and pCVD469 (kind gift of Dr. D. Karpman, Lund, Sweden) were used for recombinant expression of respectively EspA and EspB as described earlier (Karpman et al., 2002). Plasmid pMW103 (kind gift of Dr. A. O'Brien, Bethesda, U.S.) was used to express the C-terminal 380 amino acids of intimin-γ (referred to as intimin) as previously described (Sinclair and O'Brien, 2002).

1.3.6. ELISA for determination of antibodies against virulence factors of *E. coli* O157:H7

Serum samples were heat-inactivated (30 min. at 56°C) and kaolin-treated for use in ELISA tests. Polysorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 200 ng/well of intimin, EspA or EspB in PBS for 2h at 37°C and blocked overnight at 4°C with PBS + 0.2% Tween®80. After washing with PBS + 0.2% Tween®20, plates were incubated with serum samples in 2-fold dilution series, followed by isotype-specific anti-sheep HRP conjugates (anti-IgG, anti-IgA, anti-IgM, all from AbD Serotec, Düsseldorf, Germany). The buffer for dilution of samples and

conjugates was PBS + 3% BSA for the IgG ELISA, and PBS + 0.05% Tween®20 + 3% BSA for IgA and IgM ELISA tests. Each incubation step of 1h at 37°C was followed by 3 washes. After addition of 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt (ABTS) (Roche Diagnostics, Vilvoorde, Belgium), the optical density was measured at 405 nm (OD_{405}). Endpoint titres were defined as the inverse of the highest dilution where the optical density still exceeded the cut-off value, with cut-off = mean OD_{405} + 3 × standard deviation of OD_{405} values of day 0 sera from all sheep. Sera from animals intramuscularly immunized with intimin, EspA and EspB were used as positive controls.

Faecal samples and intestinal contents were diluted ½ (w/v) in PBS supplemented with 2% normal horse serum, penicillin (100 I.U./ml), streptomycin (100 µg/ml) and 0,2% Tween®20 and heat inactivated. The supernatant obtained after centrifugation was tested in ELISA as described above.

1.3.7. Lymphocyte proliferation test

Peripheral blood was collected from the jugular vein on heparin (50 I.U./ml blood) and centrifuged (1000xg at 18°C for 25 min). Peripheral blood monomorphonuclear cells (PBMC) were isolated from the buffy coat by density gradient centrifugation (800×g at 18°C for 45 min) on Lymphoprep® (Nycomed Pharma AS, Life Technologies, Merelbeke, Belgium) and collected from the interphase. After lysis of erythrocytes in ammonium chloride (74.7%) and subsequent centrifugation (380×g at 4°C for 10 min), the pelleted cells were washed and resuspended at the desired concentration in leucocyte medium (RPMI-1640 (Gibco, Life Technologies, Merelbeke, Belgium) containing FCS (10%), 2-mercaptoethanol (5×10^{-5} M), non-essential amino acids (100 mM), Na-pyruvate (100 µg/ml), L-glutamine (292 µg/ml), penicillin (100 I.U./ml), streptomycin (100 µg/ml) and kanamycin (100 µg/ml)). PBMC were seeded in triplicate at 5×10^5 cells/well in sterile 96-well plates and stimulated with 3 µg/well of intimin, EspA or EspB, 0.5 µg/well ConA as positive control or with medium alone as negative controle. After 4 days incubation at 37°C, 1 µCi 3H-thymidine was added to each well and the plates were incubated for another 18h at 37°C. Cells were harvested on glass fibre filters and the incorporated radioactivity was measured using a beta scintillation counter (Perkin-Elmer). Results are expressed as geometrical mean counts per minute (CPM).

1.3.8. Statistics

Data from the proliferation tests performed during experiment 2 were analysed with SPSS17 using one-way ANOVA with least significant difference (LSD) post hoc tests to compare the proliferative responses between different groups of animals. A p-value < 0.05 was considered statistically significant.

1.4. Results

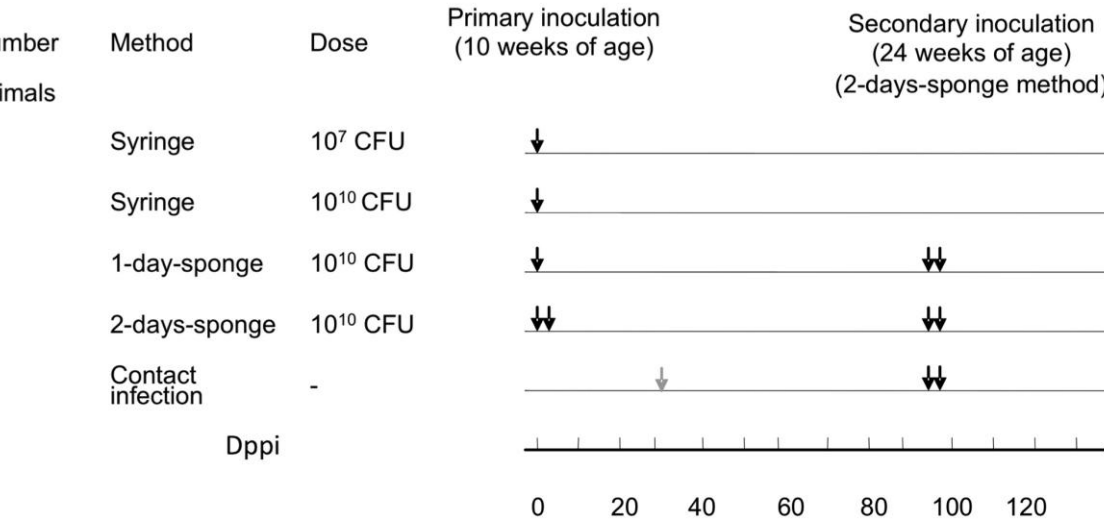
1.4.1. Excretion of *E. coli* O157:H7 following rectal inoculation of sheep

In the first experiment, 4 different rectal inoculation methods were tested. All inoculated animals excreted *E. coli* O157:H7 in the faeces during at least 16 days (data not shown). Inoculation via a syringe (group 1 and 2) resulted in variable duration of shedding, irrespective of low (16-48 days) or high dose (16-70 days). Group 3 which was inoculated once with the sponge, showed the shortest (16-27 days) excretion, whereas group 4 which received the sponge on 2 consecutive days showed the longest (48-78 days) excretion (Figure 1b). One animal in group 4 remained positive by direct enumeration up to day 55. Therefore, the 2-days-sponge method was used in further experiments. The contact-exposed animals showed excretion in low amounts (between 10^1 and 10^2 CFU/g), as shedding was only detectable by IMS (Figure 1b).

To determine if animals could be re-infected, the animals from groups 3 and 4 (respectively short and long excretion after primary inoculation) were re-inoculated at day 98 ppi (i.e. 3 weeks after ceasing of shedding in all animals) by the 2-days-sponge method. The contact-exposed animals were also inoculated at this time, to account for possible differences resulting from experimental (rectal) versus natural (oral) infection. All animals became colonized and shed for 9 to 33 days (Figure 1b), with no clear differences between the groups in terms of duration of shedding. Each individual animal in groups 3 and 4 shed an equal or lower number of days after the secondary inoculation compared to the primary inoculation.

(a)

Group	Number of animals	Method	Dose
1	3	Syringe	10^7 CFU
2	3	Syringe	10^{10} CFU
3	2	1-day-sponge	10^{10} CFU
4	2	2-days-sponge	10^{10} CFU
5	2	Contact infection	-



(b)

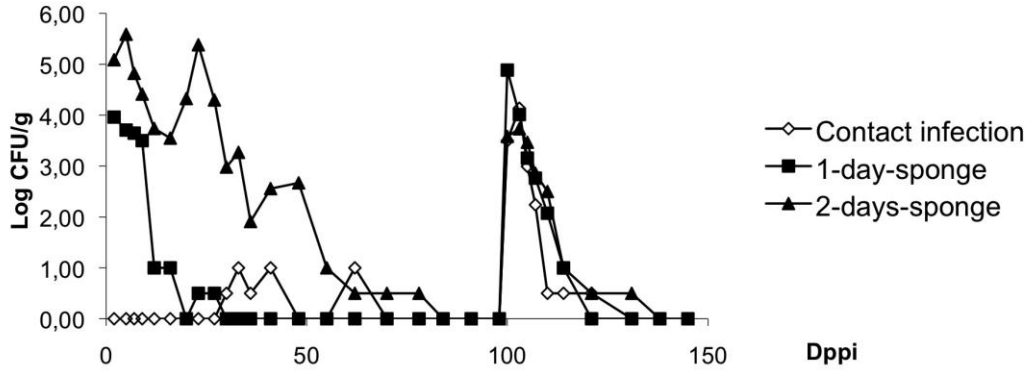


Figure 1: Experiment 1. (a) Timeline: primary inoculations were administered at 0 dppi, by different methods and doses; secundary inoculations were administered at 98 dppi by the 2-days-sponge method. Black arrows: experimental inoculation; grey arrow: natural infection by contact exposure to culture positive sheep. (b) Excretion of *E. coli* O157:H7 following primary and secondary inoculation of 3 groups of sheep by different methods. Results are presented as average values of animals in a group. CFU: colony forming units. Dppi: days post primary inoculation.

A second experiment was performed to repeat the 2-days-sponge method, to determine the localization of *E. coli* O157:H7 and to further investigate the effect of re-infection on immune responses (discussed below). Sheep were rectally inoculated by the 2-days-sponge method and excreted *E. coli* O157:H7 for 7 to 22 days. Six weeks after the primary inoculation, these animals were re-infected, and a group of previously unexposed animals was inoculated at this time to investigate the effect of age on shedding. Re-infected animals excreted during 12 to 76 days, whereas previously unexposed animals excreted during 23 to 41 days (Figure 2b). The control group was not inoculated and remained negative throughout the experiment.

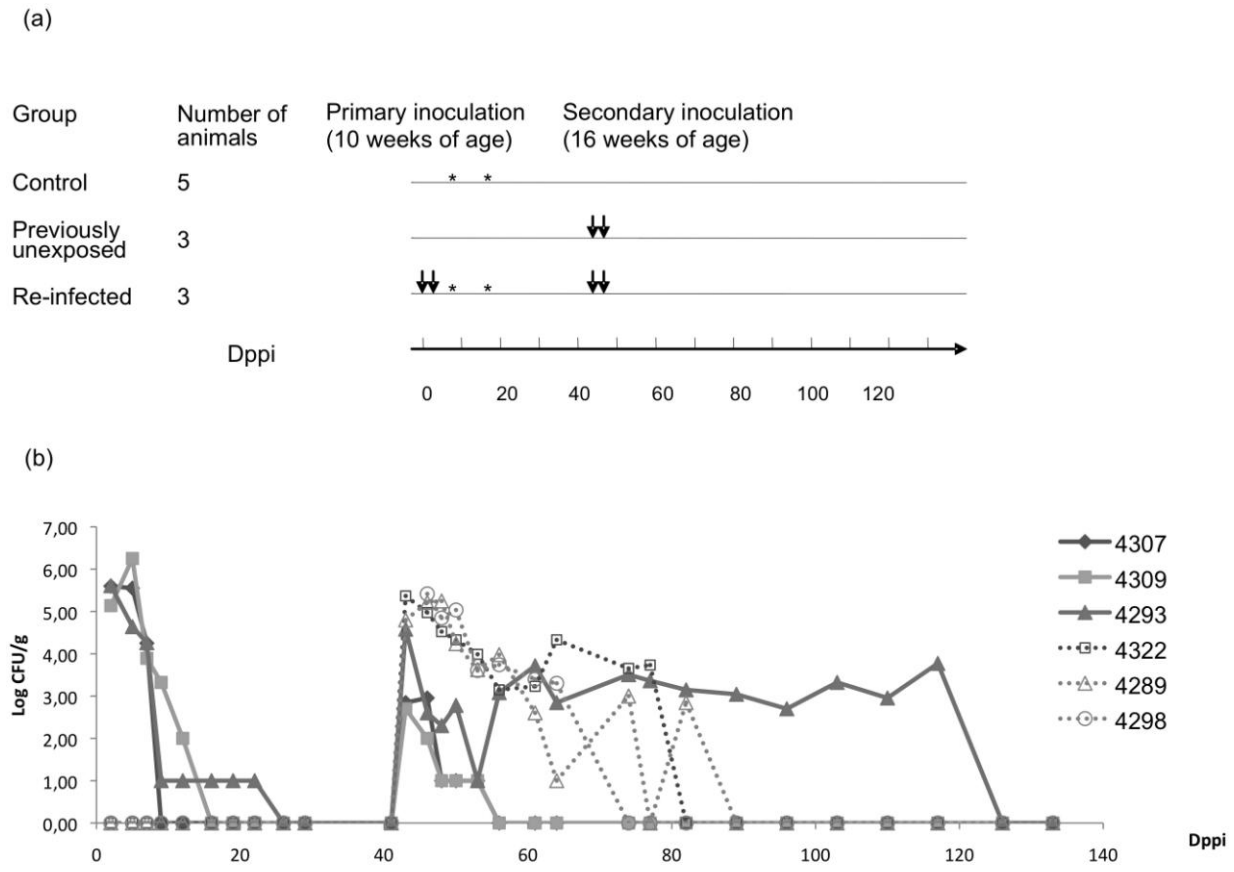


Figure 2: Experiment 2. (a) Timeline. Arrows indicate experimental inoculation, stars indicate euthanasia of one animal. (b) Excretion of *E. coli* O157:H7 following primary and secondary rectal inoculation of sheep by the 2-days-sponge method. Closed symbols represent animals from the re-infected group (animals 4307, 4309 and 4293), open symbols represent previously unexposed animals (animals 4322, 4289 and 4298). Results are presented as average values of animals in a group. Dppi: days post primary inoculation.

1.4.2. Localization of *E. coli* O157:H7 after rectal infection

In experiment 2, the intestinal localiation of *E. coli* O157:H7 was determined at 8 and 15 dppi. *E. coli* O157:H7 was recovered from the intestinal content of infected animals, and not from control animals on both time points. *E. coli* O157:H7 was found in intestinal content from the caecum, colon and rectum, but was not recovered from the small intestine as samples taken from the jejunum and ileum were negative after enrichment. Whereas rectal contents were positive by direct plate counts, with 5.00 x 10^2 CFU/g and 2.30 x 10^3 CFU/g detected on 8 and 15 dppi respectively, colon and caecum contents were only positive after enrichment culture and IMS, indicating bacterial levels lower than 10^2 CFU/g.

1.4.3. Analysis of serum antibody responses

Serum samples obtained in both experiments were tested for the presence of antibodies against intimin, EspA and EspB. Figure 3 shows the IgG response of animals infected by the 2-days- and 1-day-sponge method, and by contact exposure (experiment 1). IgG titres were low to absent, with a maximum titre of 20 in any of the animals. IgG titres in experiment 2 were similarly low and IgA or IgM were not detected in both experiments (data not shown). There was no difference between infected or non-infected animals, nor was there any effect of re-infection.

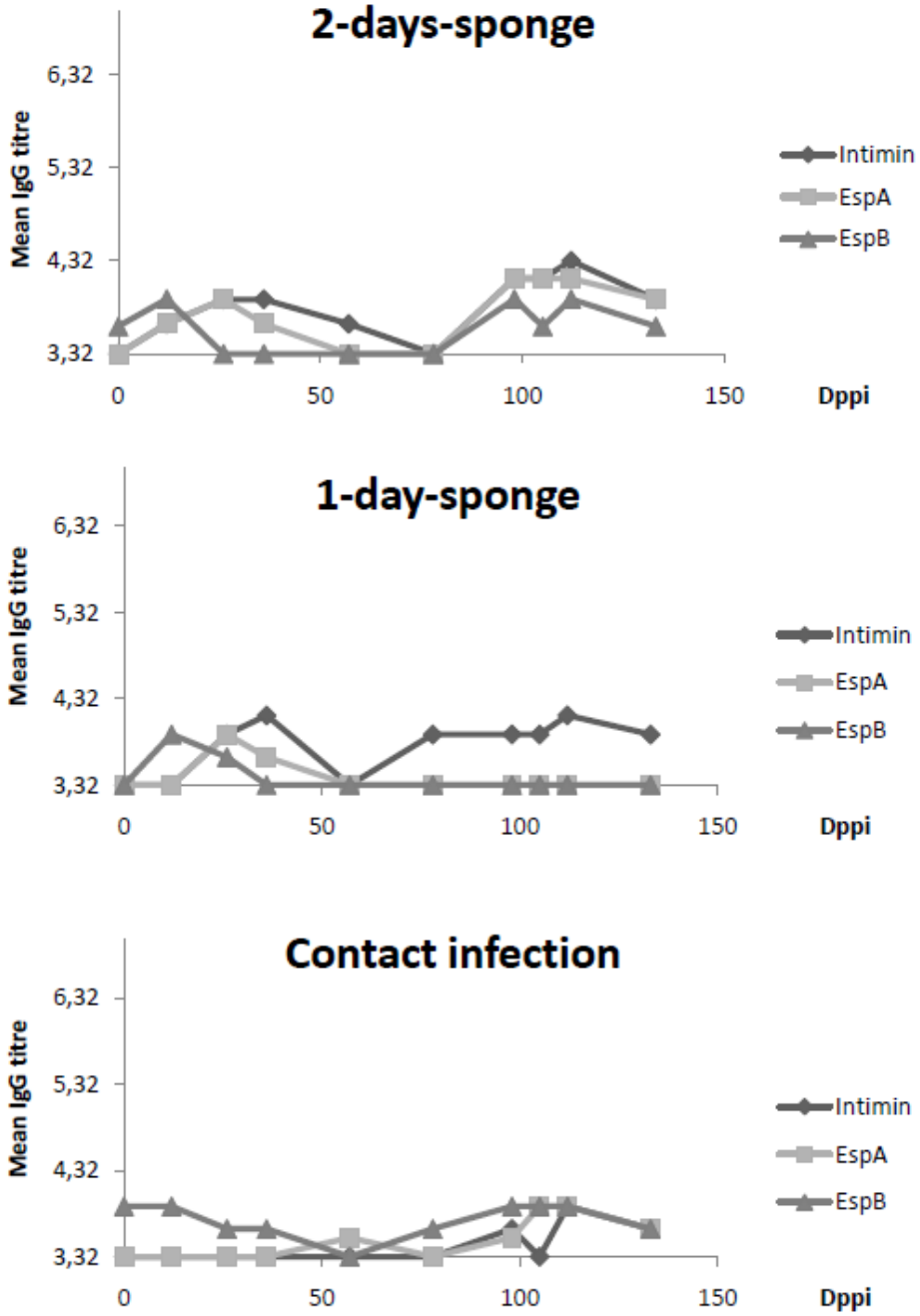


Figure 3: Serum antibody responses following rectal inoculation of animals infected by the 2-days-sponge method, 1-day-sponge method and contact exposure (experiment 1). Results are presented as average $\log_2$ values of titres.

1.4.4. Local intestinal antibody responses

Intestinal contents from rectum, colon, caecum, ileum and jejunum were tested for intimin-, EspA- and EspB-specific IgA, to determine the presence of a local immune response. However, IgA was not detected in any of the samples. Faecal antibodies were not detected either (data not shown).

1.4.5. Lymphocyte proliferation

In experiment 2, lymphocyte proliferation was measured at 9 and 16 dppi, immediately before (41 dppi) and 5 days after the secondary inoculation (46 dppi) (Figure 4). Animals in the previously unexposed group were only inoculated at 41 dppi and can be regarded as uninfected animals before 41 dppi. At 9 dppi, the proliferative responses against intimin, EspA and EspB were higher in infected animals than in uninfected animals, and this response was significant for EspA ($p < 0.05$). Two out of three infected animals were excreting *E. coli* O157:H7 at that time. At 16 dppi, all animals had stopped shedding and lymphocyte proliferation in the infected animals decreased, but remained higher than in uninfected animals, with a significant difference for the response against intimin ($p < 0.05$). At 41 dppi, responses against all antigens decreased further and re-inoculation did not enhance proliferation at 46 dppi. The previously unexposed animals were inoculated at 41 dppi, but did not show a proliferative response that was significantly different from the response in control animals at 46 dppi.

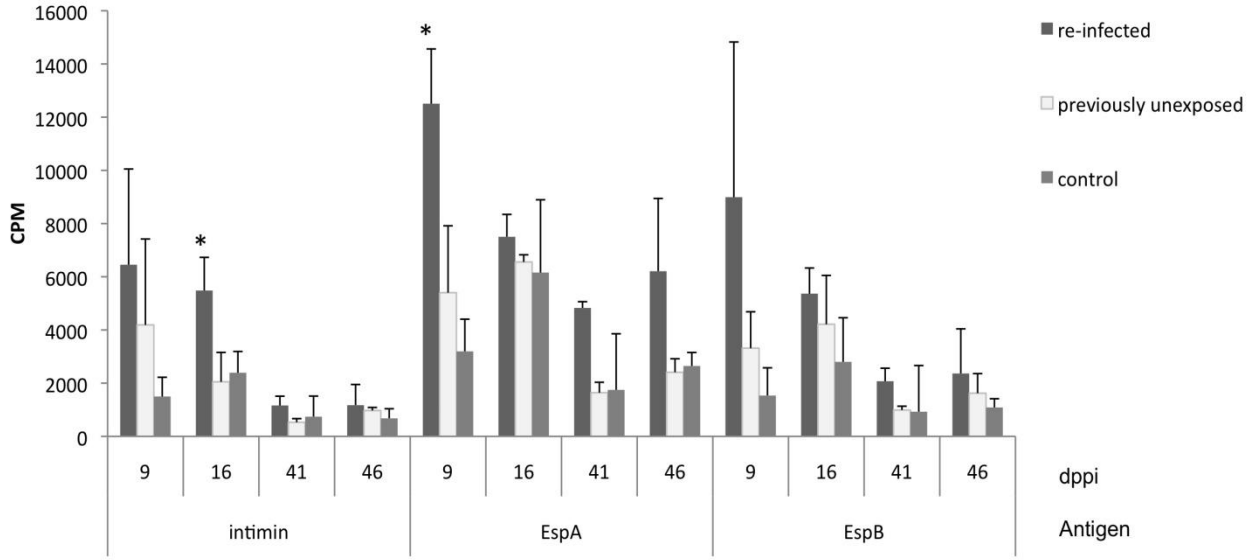


Figure 4: Lymphocyte proliferation of peripheral blood monomorphonuclear cells (PBMC) of rectally inoculated sheep after *in vitro* stimulation with virulence factors of *E. coli* O157:H7. Results are presented as counts per minute (CPM) ± SEM. *: significant differences ($p < 0.05$) between the re-infected group and the other groups.

1.5. Discussion

Our goal was to develop a rectal inoculation model leading to persistent *E. coli* O157:H7 infection of sheep, and to use this model to study immune responses occurring after infection. Other investigators have attempted rectal inoculation of cattle and sheep (Sheng et al., 2004; Best et al., 2009) for different purposes. Best et al. (2009) inoculated sheep rectally using a liquid inoculum, but they did not monitor excretion for more than 2 weeks. In the present study, excretion was monitored until it was no longer detectable by a sensitive IMS method. In the first experiment, the 2-days-sponge method resulted in long duration of excretion (48-78 days), similar to excretion patterns reported after natural infection (Kudva et al., 1997a; Widiasih et al., 2004a). Therefore, this method was used to study the immune response of sheep in the second experiment. However, even though experimental procedures were repeated exactly and animals were of the same age as in the first experiment, excretion after primary inoculation in the second experiment was of shorter duration (7-22 days). On the other hand, previously unexposed age-matched animals shed for 23 to 41 days. Thus, excretion of *E. coli* O157:H7 after rectal inoculation appears to be variable, as has been reported for oral inoculation experiments with *E. coli* O157:H7 in cattle and sheep (Cray and Moon, 1995; Sanderson et al., 1999; Cookson et al., 2002; Sheng et al., 2004).

Sheng and co-workers (2004) reported that *E. coli* O157:H7 colonization remained limited to the rectoanal junction in cattle that were rectally inoculated by swabbing the rectal mucosa. Although we applied a similar local inoculation method in sheep using a sponge, colonization extended to the distal large intestine and was not solely confined to the rectal mucosa. Our results confirm that *E. coli* O157:H7 colonises the ovine rectal mucosa less exclusively than in cattle (Best et al., 2009).

Strain NCTC12900 is a naturally occurring strain and was previously described and characterised in *in vitro* and *in vivo* models (Dibb-Fuller et al., 2001; Wales et al., 2002; Woodward et al., 2003). It does not possess Shiga toxins, but it was reported that Shiga toxin does not facilitate *E. coli* O157:H7 colonization in sheep and cattle (Sheng et al., 2006b; Cornick et al., 2007). On the other hand, it has been shown that Stx can suppress the lymphocyte proliferation response (Hoffman et al., 2006). Infection studies with Shiga toxin-positive strains are needed to further elucidate the influence of Shiga toxin and other virulence factors on immune responses of ruminants against *E. coli* O157:H7.

Sedgmen and co-workers (2002) describe the presence of lymphoid follicles in the rectal mucosa of sheep, similar to Peyer's patches, which are known induction sites of mucosal immune responses. However, we were unable to detect serum, intestinal or faecal antibodies after rectal inoculation of sheep with *E. coli* O157:H7, indicating that rectal inoculation does not induce humoral responses. Nevertheless, cellular responses as measured by proliferation tests were demonstrated early after primary infection, with a marked response against EspA and intimin. However, the existence of a cellular response did not prevent re-infection. It was speculated that reduced excretion of *E. coli* O157:H7 in cattle after re-infection can be explained by the rise of a protective immune response (Sanderson et al., 1999; Naylor et al., 2007). Our results show that *E. coli* O157 can persist in sheep as seen in cattle, with re-infection occurring after a secondary inoculation but without the rise of a clear antibody response after rectal inoculation.

As serum antibodies have been described after oral experimental infection (Johnson et al., 1996; Bretschneider et al., 2007b), it is possible that *E. coli* O157:H7 has to interact with the lymphoid tissue of the small intestine to induce clear immune responses. However, data from experimental infection studies should be carefully interpreted as they are often derived from animals with pre-existing antibody titres (Johnson et al., 1996; Bretschneider et al., 2007b). To our knowledge, this is the first study to evaluate O157-seronegative sheep combined with the study of immune responses and long-term monitoring of excretion. Rectal inoculation does not lead to humoral immune responses in young sheep, but induces cellular responses, particularly to EspA and intimin. It remains to be determined whether this cellular response is suppressing the humoral immunity and thus could explain the persistent infection of *E. coli* O157:H7.

1.6. Conclusion

Rectal inoculation of sheep with *E. coli* O157:H7 leads to infection of the lower gastrointestinal tract, in the absence of a protective immune response.

1.7. Acknowledgements

This research was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment. The authors wish to thank Dr. M. Woodward for providing strain NCTC12900, Dr. D. Karpman and Dr. A. O'Brien for providing the plasmids, Bert Driessen from the Zoological Centre in Leuven for assistance during the sheep screening, and Rudy Cooman for assistance with animal handling.

Chapter 2:

Oral infection with a Shiga toxin-negative *E. coli* O157:H7 strain elicits humoral and cellular responses but does not protect sheep from colonization with the homologous strain

Based on: Vande Walle K., De Zutter L., Cox E., 2010. Oral infection with a Shiga toxin-negative *E. coli* O157:H7 strains elicits humoral and cellular responses but does not protect sheep from colonization with the homologous strain. Veterinary Microbiology, in press, doi:10.1016/j.vetmic.2010.09.012.

2.1. Abstract

We have previously shown that rectally inoculated sheep excrete *E. coli* O157:H7 during weeks to months without developing a clear antibody response. However, antibodies against this bacterium were observed in naturally infected sheep, which most likely became orally infected. To understand this difference, sheep were orally inoculated with the same Shiga toxin-negative *E. coli* O157:H7 strain that was used for the rectal inoculation. A primary oral inoculation resulted in shedding of *E. coli* O157:H7 in the faeces and detection of antibody responses against intimin, EspA and EspB. The antibody titres waned as shedding decreased. A secondary inoculation resulted in longer shedding, even though a booster antibody response occurred. Cellular responses followed a similar pattern as the antibody levels, albeit with a lower secondary response. The presence of antigen-specific antibody-secreting cells indicates involvement of both a systemic response in the spleen and a local immune response in the terminal rectum. These results suggest that *E. coli* O157:H7 has to pass the small intestine to evoke antibody responses.

2.2. Introduction

E. coli O157:H7 is a foodborne pathogen posing an important public health risk (Rangel et al., 2005). Therefore, contamination with *E. coli* O157:H7 may have important economical consequences due to withdrawal of food from the market (Stevens et al., 2002a). Ruminants constitute the major reservoir, harbouring the bacteria in the gastrointestinal tract without showing clinical symptoms (Caprioli et al., 2005). Faecal shedding patterns of *E. coli* O157:H7 by ruminants have been extensively studied (Besser et al., 1997; Cookson et al., 2002; Naylor et al., 2007) and excretion can be detected during several months (Cray and Moon, 1995; Liebana et al., 2005). The reason for this persistence remains unclear, although several studies suggest a suppression of the host immune system by *E. coli* O157:H7. Indeed, Shiga toxin (Stx) has been observed to suppress the activation of bovine lymphocytes *in vitro* (Menge et al., 1999), and to decrease specific cellular immune responses in cattle during infection with *E. coli* O157:H7 (Hoffman et al., 2006). Furthermore, EspJ, an effector molecule inserted into the host cell by the type III secretion system, is known to inhibit opsono-phagocytosis of *E. coli* O157:H7 by macrophages (Marches et al., 2008). In addition, *E. coli* O157:H7 contains a functional homologue of lymphostatin (Abu-Median et al., 2006), a toxin of the related enteropathogenic *E. coli* (EPEC), which inhibits human lymphocyte proliferation and cytokine expression in

response to mitogen stimulation (Klapproth et al., 2000). Moreover, Hu and co-workers (2009) recently showed a specific block in antibody secretion by the subtilase cytotoxin of *E. coli* O157:H7. However, only limited *in vivo* data are available (Hoffman et al., 2006). The aim of our research is to study the immune response of sheep against *E. coli* O157:H7. We previously demonstrated that rectal inoculation with *E. coli* O157:H7 induced a cellular immune response without evoking antibody responses against intimin, EspA or EspB even though the bacteria were excreted during a long period of time (48 to 78 days) (Vande Walle et al., 2010a). However, antibodies against these proteins were observed in the serum of naturally infected sheep (Vande Walle et al., unpublished data). Therefore, in the present study we analysed excretion and immune responses against *E. coli* O157:H7 following oral inoculation, which resembles more the natural route of infection.

2.3. Material and methods

2.3.1. Bacterial inoculums

NCTC12900 is a well characterized Stx-negative *E. coli* O157:H7 strain with spontaneous nalidixic acid resistance (Dibb-Fuller et al., 2001; Wales et al., 2002; Woodward et al., 2003). For preparation of bacterial inocula, NCTC12900 was grown overnight in Luria Bertani broth at 37°C while shaking (200 rpm), washed with sterile phosphate-buffered saline (PBS) and resuspended in 10% saccharose (Merck) solution.

2.3.2. Experimental animals and procedures

Eight ten-week-old conventionally reared sheep (Belgian cross-breed, Zootechnical Centre, Leuven, Belgium), culture negative for *E. coli* O157:H7 in faeces (on 2 separate occasions) and seronegative for antibodies against intimin, EspA and EspB, were allowed to acclimatize for one week after arrival in our facilities. Sheep received rye-grass silage supplemented with a grain-based pelleted diet (mainly barley, corn, alfalfa and oat) and water *ad libitum*. The sheep were housed in groups with four animals per pen.

After the acclimatization period, lambs were orally given colistin at 50.000 I.U./kg body weight (Promycine pulvis 1 million I.U./g, VMD, Berendonk, Belgium), twice daily during 5 days, to prevent *E. coli* infections due to handling and transport of the animals. Prior to the inoculation (2 days after the last colistin dose), lambs were given a 10% $NaHCO_3$ solution via a nursing bottle, to close the oesophageal groove in order to direct the inoculum into the abomasum. Four animals were inoculated orally with 10^{10} CFU of NCTC12900. The inoculum was given on two

consecutive days, as for the rectal infection model (Vande Walle et al., 2010). The other four lambs served as a negative controls and received the $NaHCO_3$ and saccharose solution but not the inoculum. Faecal excretion of NCTC12900 was monitored by direct plating and immunomagnetic separation (IMS) as described (Vande Walle et al., 2010a). Excretion was initially monitored three times per week, and this sampling frequency was gradually reduced to once a week as shedding decreased. Thirty-six days after ceasing of shedding in the longest shedding animal (66 days post primary inoculation, dppi), the four lambs were re-inoculated in a way identical to the primary inoculation, to determine the presence of protective immunity. Recombinant intimin, EspA and EspB were produced as described to analyse the immune responses against these virulence factors (Karpman et al., 2002; Sinclair and O'Brien, 2002; Vande Walle et al., 2010a). Serum was sampled weekly to monitor antibody responses by ELISA (Vande Walle et al., 2010a) and blood on days 17, 38, 56, 76, 85 and 100 post primary inoculation (dppi) to monitor cellular immune responses. One animal was euthanized 40 days after the secondary inoculation (106 dppi) to determine the intestinal localization of NCTC12900. Hereto, intestinal contents and tissues (rumen, duodenum, jejunum, ileum, caecum, colon and rectum) were tested for the presence of NCTC12900 by direct plate counts and IMS as described for faecal samples. Before testing, tissues were carefully rinsed with sterile PBS to remove non-adherent bacteria. Intestinal contents were also tested in ELISA for the presence of antibodies (Vande Walle et al., 2010a). Three animals were euthanized 74 days after the secondary inoculation (140 dppi) when all animals had stopped shedding to localize the immune response by determining antibodies in intestinal contents and the number of antibody-secreting cells (ASC) in different tissues: spleen, mesenterial (MLN), colonic (CoLN) and rectal lymph nodes (RLN), Peyer's patches from the jejunum (JPP) and ileum (IPP), lamina propria of the jejunum (LPJ) and the rectum (LPR). The remaining animals were euthanized at 140 dppi without further sampling. All experimental and animal management procedures were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

2.3.3. Evaluation of the cellular immune response: proliferation test

Peripheral blood monomorphonuclear cells (PBMC) were isolated from blood as described (Vande Walle et al., 2010a), seeded in triplicate at 5 x 10^5 cells/well in sterile 96-well plates and stimulated with 100 µg/ml intimin, 100 µg/ml EspA and 25 µg/ml EspB. After 4 days incubation at 37°C, 1 µCi ^{3}H-thymidine was added to each well and the plates were incubated for another 18h at 37°C. Cells were harvested on glass fibre filters and incorporated radioactivity

was measured using a beta scintillation counter (Perkin-Elmer, Brussels, Belgium). Results are expressed as stimulation indices (SI): geometric mean of stimulated wells/geometric mean of unstimulated wells. Stimulation with 0.5 µg/well ConA and cell culture medium served as positive and negative control, respectively.

2.3.4. Isolation of monomorphonuclear cells (MC) from spleen, lymph nodes and intestine

MC from spleen, MLN, CoLN, RLN, JPP and IPP were isolated essentially as described by Van den Broeck et al. (1999). Intestinal segments from the LPJ and LPR were washed with PBS + 100 I.U./ml penicillin + 100 µg/ml streptomycin, and the serosa and the muscle layer were removed. The tissue was cut into 2-3 mm^2 pieces and incubated three times for 40 minutes in Hank's balanced salt solution (HBSS) with 0.94 M dithiotreitol, 2.52 M EDTA and 100 I.U./ml penicillin and 100 µg/ml streptomycin in a shaking incubator at 37°C followed by sieving the intestinal fragments. After rinsing the tissue with HBSS, it was incubated during 30 minutes in RPMI with 5% FCS, 20 mM HEPES, 0.1 mg/ml DNase I, 100 I.U./ml penicillin and 100 µg/ml streptomycin. Subsequently, the tissue fragments were incubated for 1h in the RPMI solution with 0.36 mg/ml collagenase A in a shaking incubator at 37°C. Then, the cell clumps were removed by filtration through a 70 µm cell strainer. The obtained MC from the different tissues were washed with PBS + 1 mM EDTA. Cells were resuspended in leucocyte medium at 5 x 10^6 cells/ml.

2.3.5. ELISPOT assay for antigen-specific IgG, IgA and IgM ASC

Antigen-coated plates were prepared as described for the ELISA tests (Vande Walle et al., 2010a). In addition to intimin, EspA and EspB, plates were coated with 100 µl/well of a sonicated NCTC12900 suspension (5 x 10^8 CFU/ml, further referred to as O157 lysate). Thereafter, MC suspensions at a concentration of 5 x 10^6 cells/ml were added (100 µl/well) and plates were incubated for 16 h at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, the cells were removed by ten washes with ELISA washing buffer and wells were incubated for 1h at 37°C with anti-sheep IgG-, IgA- or IgM- HRP conjugated secondary antibody (all from AbD Serotec, Düsseldorf, Germany). After incubation, the plates were washed 3 times with ELISA washing buffer. Next, a substrate solution, consisting of 4 volumes of 3-amino-9-ethylcarbazole (AEC) (Sigma, Bornem, Belgium) working solution (0,67 ml AEC stock solution (0,4% in dimethylformamide) in 10 ml Na acetate (0,1 M, pH 5,2) + 10 µl 30% H_2O_2) and 1 volume of 3%

low-melting-point agarose (BIOzym, Landgraaf, The Netherlands) was added. After overnight incubation in the dark at room temperature, spots were counted with an ELISPOT reader (Immunospot, CTL). For each MC suspension, spots in 5 wells were counted to obtain the number of isotype-specific ASC/2,5x10^6 MC.

2.4. Results

2.4.1. Excretion and intestinal localization of *E. coli* O157:H7

Following the primary inoculation, excretion of *E. coli* O157:H7 in the 4 orally inoculated lambs lasted minimally 13 and maximally 30 days (Figure 1). Excretion was highest 3 to 10 days after inoculation and declined rapidly thereafter. The lambs were re-infected 36 days after ceasing of excretion in all animals (66 dppi). Excretion after this secondary inoculation lasted consistently longer than after the primary inoculation, varying from 28 to 69 days. Animal 4532 was euthanized 40 days after the secondary inoculation (106 dppi), and was still excreting *E. coli* O157:H7 at that time. *E. coli* O157:H7 could be recovered from all sites throughout the intestine: rumen, duodenum, jejunum, ileum, colon, caecum and rectum. Intestinal content and tissue parts from all sites except the rectum were only positive after enrichment culture, indicating bacterial counts between 10^1 and 10^2 CFU/g. However, intestinal content of the rectum was positive for *E. coli* O157:H7 in direct plate counts, with 1.035 x 10^4 CFU/g.

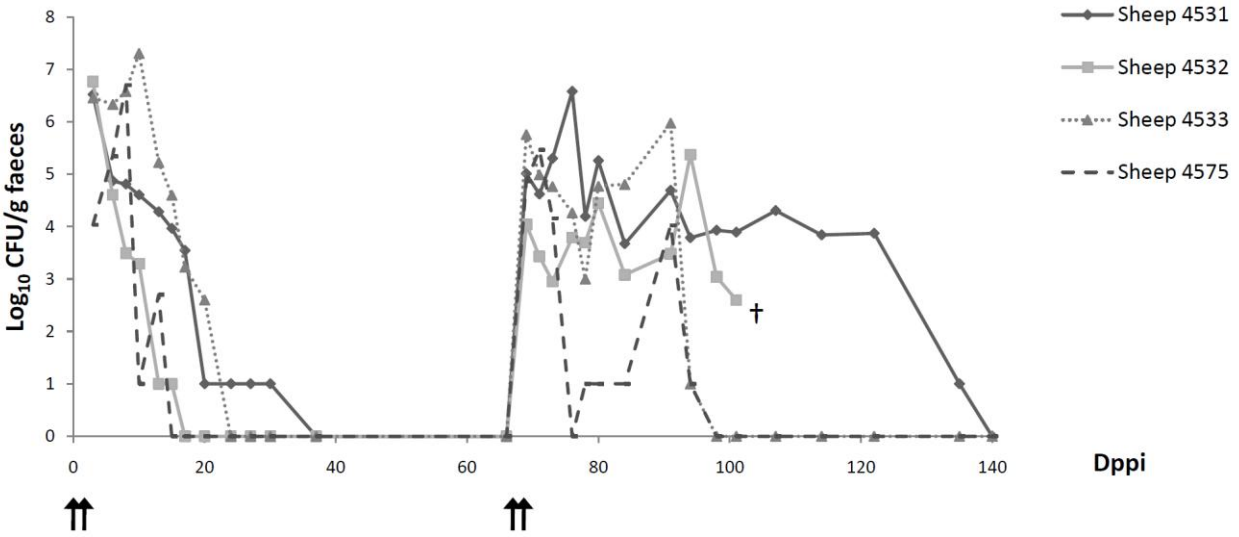


Figure 1: Excretion of *E. coli* O157:H7 of orally inoculated sheep. Results are presented as $\log_{10}$ values of colony forming units (CFU)/g faeces. Arrows indicate time of inoculations. *E. coli* O157:H7 was never recovered from any of the control animals. † euthanasia of animal 4532 at 106 dppi. Dppi: days post primary inoculation.

2.4.2. Antibody responses

Serum was sampled weekly to evaluate the antibody responses against intimin, EspA and EspB. The infected animals responded against the infection by producing IgG against all three antigens (Figure 2). The IgG titres reached a peak around 3 weeks post primary inoculation and decreased as the shedding levels declined (Figure 1). Following the secondary inoculation the titres against EspA and EspB rapidly rose again, indicating a secondary immune response. This was most pronounced for EspA whereas only a weak response was observed against intimin. These responses could not be considered as real secondary responses, which are characterized by higher titres and occur faster after contact with antigens than a primary response. Overall, animals 4531 and 4533 responded with the highest antibody titres whereas animals 4532 and 4575 only showed low responses (individual data not shown). Serum IgM or IgA antibodies were not detected in any of the animals and the control animals remained seronegative throughout the experiment (data not shown).

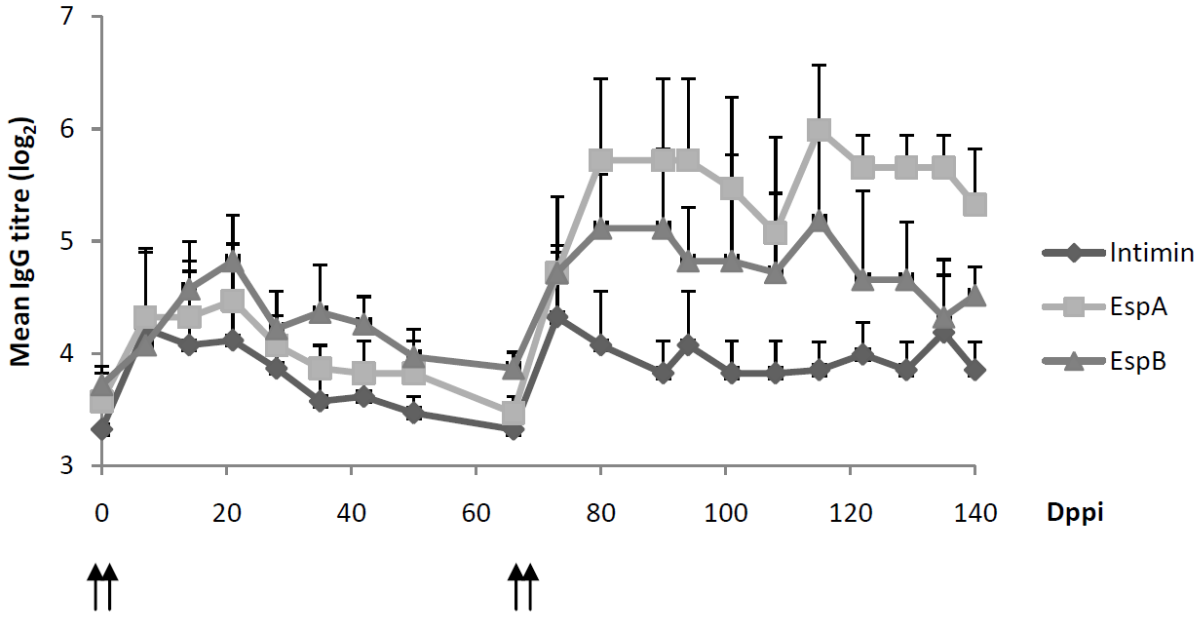


Figure 2: Serum IgG antibody responses of orally inoculated sheep against intimin, EspA and EspB. Results are presented as log_2 titers ± SEM. Arrows in icate time of inoculations. Dppi: days post primary inoculation.

To localize and quantify the antibody response following EHEC infection, two infected animals that showed clear serum antibody responses (4531 and 4533) and one control animal were euthanized at 140 dppi. Antigen-specific IgG or IgA could not be detected in the intestinal contents. However, antigen-specific ASC were detected in the spleen and the rectal lamina propria of the infected animals (Table 1) but not in the other tissues. Animal 4531 which had stopped excreting *E. coli* O157:H7 five days before euthanasia, showed a clear IgM and IgG

response in the spleen and an IgA response in the rectal lamina propria that was stronger than the IgG response at this site. Animal 4533 which ceased excreting 41 days earlier, showed lower responses with only two O157-specific IgG ASC in the spleen and two IgG and six IgA O157-specific cells, probably reflecting the waning immune response due to the longer absence of infection.

Table 1: Number of antigen-specific ASC per 2.5 x 10^6 cells in different tissues of infected animals and a control animal, euthanized at 140 days post primary inoculation.

		IgG				*IgA*				*IgM*			
Animal	Tissue	Int	A	B	O157	Int	A	B	O157	Int	A	B	O157
4531	Spleen	7	+	6	0	0	+	0	1	11	17	0	7
	LPR	5	0	0	24	19	0	0	46	0	0	0	0
4533	Spleen	0	0	0	2	0	0	0	0	0	0	0	0
	LPR	0	0	0	2	0	0	0	6	0	0	0	0
Control	Spleen	0	0	0	0	0	0	0	0	0	0	0	0
	LPR	0	0	2	0	0	0	0	0	0	0	0	0

Int: intimin, A: EspA, B: EspB, O157: lysate of *E. coli* O157:H7, +: unquantifiable colour reaction (different from background), LPR: lamina propria of the rectum.

2.4.3. Lymphocyte proliferation

To analyse cellular responses, PBMC isolated at different time points after primary and secondary inoculation were restimulated *in vitro* with intimin, EspA and EspB (Figure 3). Proliferative responses against intimin were high at 17 dppi, whereafter this response gradually declined. Re-infection at 66 dppi did not result in an increase of the intimin-specific proliferation. Responses after stimulation with EspA and EspB were highest at 38 dppi, when shedding had already ceased in all animals. Re-infection seemed to cause a second rise in these responses, although they were lower than at 38 dppi. However, the control animals also showed proliferation after stimulation with EspA and EspB although *E. coli* O157:H7 excretion or serum responses were not detected. This suggests that cross-reacting antigens primed the immune system.

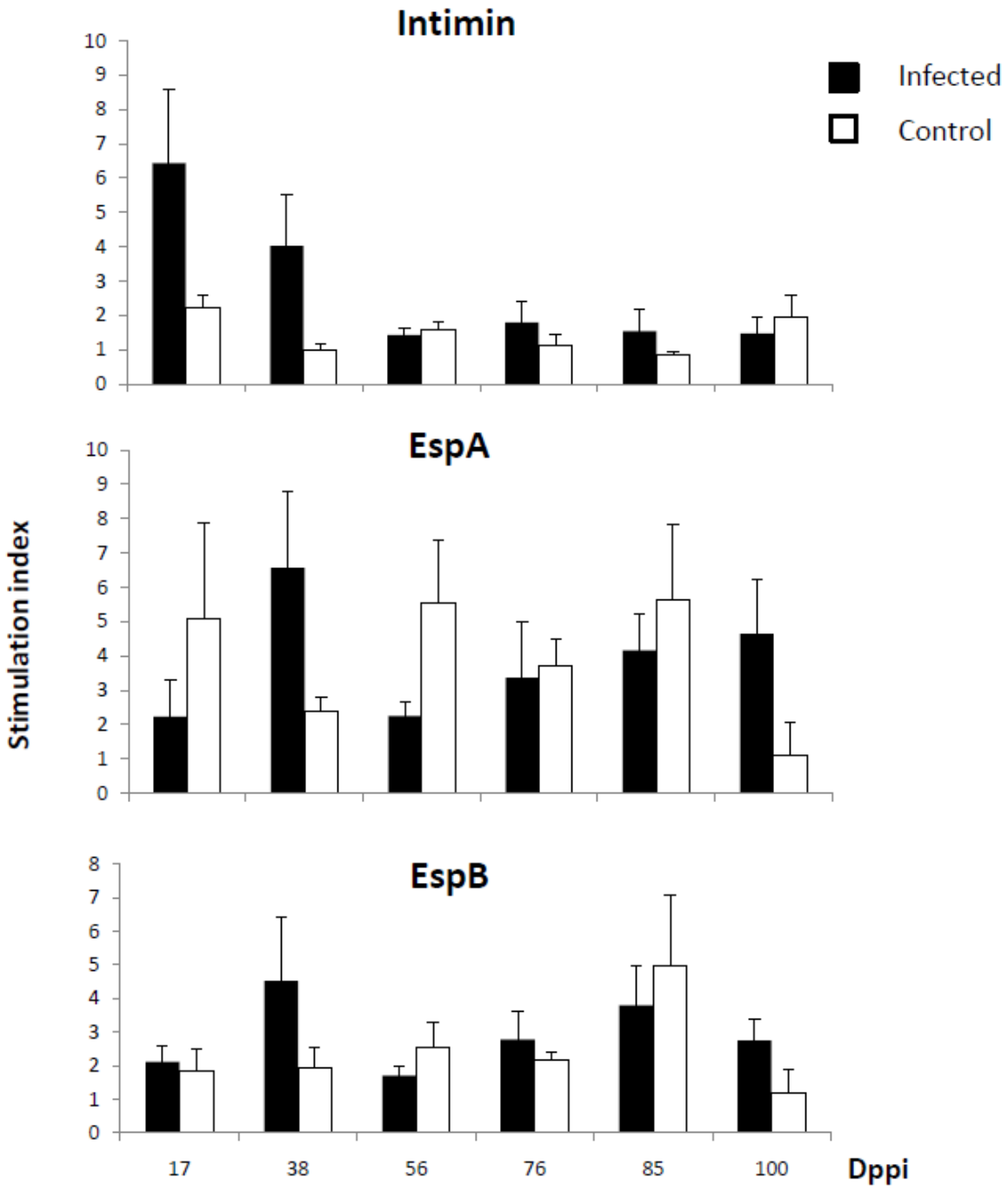


Figure 3: Lymphocyte proliferation after stimulation with intimin, EspA and EspB on different time points after oral infection. Inoculations were performed on days 0/1 and 66/67. Results are presented as stimulation index ± SEM. Black bars: average of values of infected animals (n=4), white bars: average of values of control animals (n=4). Arrows indicate time of inoculations. Dppi: days post primary inoculation.

2.5. Discussion

We used an oral infection model to analyse immune responses against *E. coli* O157:H7 and compared the results with a previously described rectal inoculation model (Vande Walle et al., 2010a). Although antibody responses were only present following oral inoculation, cellular responses were present following both rectal and oral inoculation (Vande Walle et al., 2010a and this study). In the present study, proliferative responses were high shortly after the primary inoculation and decreased as shedding disappeared. The results suggest that the cellular response against intimin could be more specific for an *E. coli* O157:H7 infection than the response against EspA and EspB, as the control animals showed a reaction against the latter two proteins in the absence of an identifiable *E. coli* O157:H7 infection or antibody response. However, the intimin-specific cellular response was not evoked by the secondary inoculation, whereas this was the case for the responses against EspA and EspB. Therefore, it cannot be excluded that contact with cross-reacting antigens provoked this secondary response. Indeed, EspA and EspB are not specific for EHEC, and are present in e.g. STEC and EPEC with high amino acid-similarity between EHEC and EPEC proteins (Abe et al., 1997; Ebel et al., 1998). Data regarding the cellular immune response against *E. coli* O157:H7 in ruminants are sparse. The only study performed so far in cattle reported a suppression of lymphocyte proliferation against heat-killed O157-antigens, mediated by Stx (Hoffman et al., 2006). In the present study, we infected sheep with an Stx-negative strain and did not find indications for lymphocyte suppression since proliferative and antibody responses were present after the primary and secondary inoculation. However, to obtain insight in the role of Stx, experiments with Stx-positive strains should be performed.

Whereas serum or intestinal antibodies were not detected after rectal inoculation even though excretion of *E. coli* O157:H7 persisted for several weeks to months (Vande Walle et al., 2010a), in the present study a clear antibody response was seen in orally inoculated sheep. Antibody titres against intimin, EspA and EspB increased after the primary inoculation, and decreased as shedding diminished. A higher secondary response occurred against EspA and to a lesser extent against EspB after the secondary inoculation, with sustained antibody titres even though shedding ceased. Since the infected animals could be recolonized, these data suggest that the antibody response was not protective or that mucosal protection was limited. This was reflected by the observation that one infected animal that only recently ceased shedding showed antigen-specific IgA and IgG ASC in the lamina propria of the rectum, whereas another

animal that ceased shedding several weeks before barely showed ASC. Moreover, the antibody response in the intestinal tract seems to be quite local as antigen-specific ASC were only detected in the rectal mucosa, and not in the draining lymph nodes or in the small intestine. However, significant amounts of antigen apparently reach the systemic immune system as reflected by the presence of antigen-specific ASC in the spleen and serum antibody responses.

Whereas after rectal inoculation *E. coli* O157:H7 was present only in the lower gastrointestinal tract (Vande Walle et al., 2010a), it could be found throughout the entire intestinal canal after oral infection. Our results indicate that in sheep colonization of the small intestine or interaction with the lymphoid tissue in the small intestine is necessary for a clear antibody response, but also seem to suggest that this antibody response is not involved in protection against infection. Peyer's patches in the small intestine are known induction sites of mucosal immune responses. At the moment it is not clear why we could not find *E. coli* O157:H7-specific ASC in other intestinal tissues than the rectal lamina propria and if this localized response explains the absence of correlation between antibody response and protection. It should be noted that at the time the local immune response was assessed, shedding had already stopped in all animals. Testing at different time points following infection would be interesting to provide a more complete and clearer picture of the ongoing immune response. Our results concur with the studies performed in cattle experimentally inoculated with *E. coli* O157:H7 where infiltration of granulocytes in the rectal lamina propria and rectal IgA antibodies recognizing several O157 antigens were demonstrated (Bretschneider et al., 2008; Nart et al., 2008a; Nart et al., 2008b). As shown before in cattle and now in sheep, pre-existing serum antibodies, whether from natural exposure or experimental infection, are not sufficient for protection (Johnson et al., 1996; Bretschneider et al., 2007b, this study). The role of the immune response during the course of infection and during clearance remains to be determined. To our knowledge, the rectal and oral inoculation studies performed in our lab (Vande Walle et al., 2010a and this study), are the first studies to investigate immune responses of sheep against *E. coli* O157:H7.

2.6. Acknowledgements

This research was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment. The authors wish to thank Bert Driessen from the Zoological Centre in Leuven for help during screening of the sheep, and Rudy Cooman, Griet De Smet and Denise Slos for excellent technical assistance.

Chapter 3:

A preliminary investigation of the role of the immune system in clearance of *E. coli* O157:H7 infection in sheep

Based on: Vande Walle K., Entrican G., Wattegedera S., De Zutter L., Vanrompay D., Cox E. A preliminary investigation of the role of the immune system in clearance of *E. coli* O157:H7 infection in sheep. Manuscript in preparation.

3.1 Abstract

Previously, we demonstrated a serum antibody response and a cellular immune response against intimin, EspA and EspB following oral inoculation of sheep with *E. coli* O157:H7. However, these responses were not protective as sheep could become re-infected by the same strain. To investigate the role of the immune response in clearance of *E. coli* O157:H7, previously infected sheep were re-infected and euthanized when shedding declined. At that time, the systemic and the intestinal mucosal immune response against intimin, EspA, EspB and Tir were analysed. A subgroup of the infected animals had first been vaccinated with a mixture of these four antigens, to investigate the influence of preceding immunization. ELISPOT tests revealed mucosal antibody responses against EspA, in the jejunal Peyer's patches of vaccinated animals and in the ileal Peyer's patches of non-vaccinated animals. Following restimulation with the antigens, the regulatory cytokine IL-12 and the Th1 cytokine IFN-γ were only produced by spleen and lymph node cells, whereas monomorphonuclear cells from the Peyer's patches and the rectal mucosa produced very low amounts or none of these cytokines. The pro-inflammatory cytokine TNF-α was produced by cells of all isolated tissues, but compared to control animals secretion was significantly lower by several cell types from non-vaccinated animals and by some cell types from vaccinated animals. The anti-inflammatory cytokine IL-10 showed the tendency to be higher in spleen, mesenteric and rectal lymph nodes of non-vaccinated animals whereas production was lower in mesenteric and rectal lymph nodes, and jejunal Peyer's patches of the vaccinated animals. The results could suggest that infection with *E. coli* O157:H7 suppresses the inflammatory response by enhancing IL-10 production. Further research is needed to elucidate the interaction of *E. coli* O157:H7 with the mucosal immune system.

3.2 Introduction

E. coli O157:H7 is a foodborne pathogen transmitted by healthy ruminants. The bacteria colonize the gastrointestinal tract and are excreted in the faeces of ruminants, without overt symptoms (Paton and Paton, 1998). The absence of serious illness such as haemorrhagic colitis and the haemolytic uremic syndrome as seen in humans is attributed to the lack of the receptor for Shiga toxin on the microvasculature of ruminants (Pruimboom-Brees et al., 2000). Shedding of *E. coli* O157:H7 by ruminants is typically intermittent as demonstrated in longitudinal studies of excretion by naturally infected cattle (Robinson et al., 2004; Smith et al., 2010) but can be detected up to several months in individual animals following both natural and experimental

infection (Besser et al., 1997; Cornick et al., 2000, Vande Walle et al., 2010a; Vande Walle et al., 2010b). In experimental infections, persistent infection is often defined as excretion for more than 14 days (Cookson et al., 2002; Woodward et al., 2003). The typical attaching and effacing (A/E) lesions that are formed in the human intestine upon colonisation, are only rarely observed in experimentally inoculated, persistently colonized animals (Wales et al., 2001a; Cookson et al., 2002). Nevertheless, strains deficient in A/E colonization factors such as intimin and Tir, are less able to persist than the wild type strains (Cornick et al., 2002; Sheng et al., 2006b; Vlisidou et al., 2006a; Bretschneider et al., 2007a), indicating important roles for these molecules in colonization.

It has been suggested that *E. coli* O157:H7 can suppress the immune system of the ruminant host, thereby facilitating persistence in these species (Menge et al., 1999; Hoffman et al., 2006; Naylor et al., 2007). Nevertheless, immune responses against various proteins of *E. coli* O157:H7 have been demonstrated by us and others (Bretschneider et al., 2007b; Nart et al., 2008a). We have shown that *E. coli* O157:H7 has to pass the small intestine to evoke antibody responses, as these responses were only observed after oral inoculation and not after rectal inoculation of sheep (Vande Walle et al., 2010a; Vande Walle et al., 2010b). However, in both cases cellular responses were present, which followed the course of infection. Neither antibody or cellular responses were protective, since sheep could be re-infected with the same *E. coli* O157:H7 strain. Nevertheless, eventually the shedding ceases and the infection is cleared. The factors leading to spontaneous clearance are unknown. The goal of the present study was to investigate which systemic or mucosal immune responses occur at the moment shedding ceases, in other words, what is the influence of the immune system on clearance of an *E. coli* O157:H7 infection. In addition, because vaccination with *E. coli* O157:H7 colonisation factors is a possible strategy to decrease shedding in cattle and sheep (Potter et al., 2004; Atef Yekta et al., 2010) we investigated the influence of a preceding systemic immunization with intimin, EspA, EspB and Tir on the immune response and clearance of infection.

3.3 Materials and methods

3.3.1 Experimental animals and procedures

A total of eleven conventionally reared sheep (Belgian cross-breed, Zootechnical Centre, Leuven, Belgium), negative for *E. coli* O157:H7 in faeces and seronegative for antibodies against intimin, EspA and EspB, were used in this experiment. The animals received hay supplemented

with a pelleted diet and water *ad libitum*. Seven of these sheep had previously been infected with the Shiga toxin-negative *E. coli* O157:H7 strain NCTC12900 (according to the method described by Vande Walle et al., 2010b), and three out of these seven sheep had first been intramuscularly immunized in the neck with intimin, EspA and EspB (100 µg each), formulated in incomplete Freund's adjuvant (Atef Yekta et al., 2010). Booster immunizations with the same antigen mixture to which Tir was added were given 5 and 1 weeks before the experimental inoculation. This sequence of immunization, infection, and booster immunizations induced high serum antibody titres in these three sheep, which we considered as hyperimmunized animals. The seven previously infected sheep were orally re-infected with 10^{10} CFU of NCTC12900, on two consecutive days as described earlier (Vande Walle et al., 2010b). Four sheep served as negative controls and were not inoculated. An overview of the immunizations and experimental infections is presented in Table 1. At 5 days post inoculation (dpi), blood was taken to determine the presence of recirculating antibody-secreting cells (ASC). Weekly serum samples were taken to monitor the antibody response. On 0 and 19 days post infection (dpi) blood was taken to determine antigen-specific proliferation. Excretion of NCTC12900 was monitored in faeces twice weekly by direct plate counts and immunomagnetic separation (IMS) as described (Vande Walle et al., 2010a). When excretion was no longer detectable by direct plate counts, animals were euthanized to analyse the immune response. Colony counts were $\log_{10}$ transformed for data analysis and used to calculate area under the curve (AUC) values by the trapezoidal method (Naylor et al., 2007) to estimate the total amount of shedding during the monitoring period following re-infection. All experimental and animal management procedures were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (n° 2009/074).

Table 1: Experimental design: overview of immunizations and infections.

Group	n	First experiment (age sheep 4 months) (Atef Yekta et al., 2010)		Second experiment (age sheep 8 months) (this study)	
Control	4	-	-	-	-
Vaccinated group	3	3x immunization with IAB	O157	2x immunization with IABT	O157
Non-vaccinated group	4	-	O157	-	O157

IAB: immunization with intimin, EspA and EspB
IABT: immunization with intimin, EspA, EspB and Tir
O157: inoculation with *E. coli* O157:H7, strain NCTC12900

3.3.2 Recombinant proteins

Intimin, EspA and EspB were produced as described (Karpman et al., 2002; Sinclair and O'Brien, 2002). Plasmid pET22bTir103 (kind gift of Dr. K. Gough, Leicester, U.K.) was used to express the intimin-binding domain of Tir (referred to as Tir) as described (Kuhne et al., 2004).

3.3.3 ELISA for determination of antibodies against intimin, EspA, EspB and Tir

ELISA assays for intimin, EspA and EspB were performed as described (Vande Walle et al., 2010a), and an assay for Tir was developed with identical conditions.

3.3.4 Antigen-specific proliferation assay

Peripheral blood monomorphonuclear cells (PBMC) were isolated from blood and used in proliferation tests as described (Vande Walle et al., 2010b). PBMC were stimulated *in vitro* with 100 µg/ml intimin, 100 µg/ml EspA, 25 µg/ml EspB or 100 µg/ml Tir. Results are expressed as stimulation indices (SI): geometric mean of stimulated wells/geometric mean of unstimulated wells. Stimulation with 0.5 µg/well ConA and cell culture medium served as positive and negative control, respectively.

3.3.5 ELISPOT assay for antigen-specific IgG, IgA and IgM antibody-secreting cells (ASC)

PBMC and monomorphonuclear cells (MC) from spleen, mesenteric (MLN), colonic (CoLN) and rectal lymph nodes (RLN), Peyer's patches from the jejunum (JPP) and ileum (IPP), and lamina propria of the rectum (LPR) were isolated, resuspended at 1 x 10^6 cells/ml and used in ELISPOT assays to detect intimin-, EspA-, EspB- and Tir-specific ASC (Vande Walle et al., 2010b).

3.3.6 Cytokine production following *in vitro* restimulation of MC

MC were seeded in triplicate at 1 x 10^6 cells/well in sterile 96-well plates and incubated with purified bacterial antigens: 100 µg/ml intimin, 100 µg/ml EspA, 25 µg/ml EspB, or 100 µg/ml Tir. The supernatant was collected after 72h incubation at 37°C in a humidified atmosphere. A TNF-α ELISA was performed with coating and capture antibodies from Perbio (Pierce, Erembodegem, Belgium) and ELISAs for IL-4, IL-10, IL-12 and IFN-γ were performed with antibodies from AbD Serotec (Düsseldorf, Germany) according to protocols used at the Moredun Institute (Hope et al., 2002; Kwong et al., 2002; Hope et al., 2005, S. Wattegedera, personal

communication). Quantification was performed using a standard curve generated using known concentrations of recombinant ovine cytokines (Moredun Institute, Edinburgh, Scotland). The cytokine concentrations were calculated using DeltaSOFT JV 2.1.2 software (BioMetallics, Princeton, NY, USA) with a 4-parameter curve-fitting algorithm applied for standard curve calculations.

3.3.7 Statistics

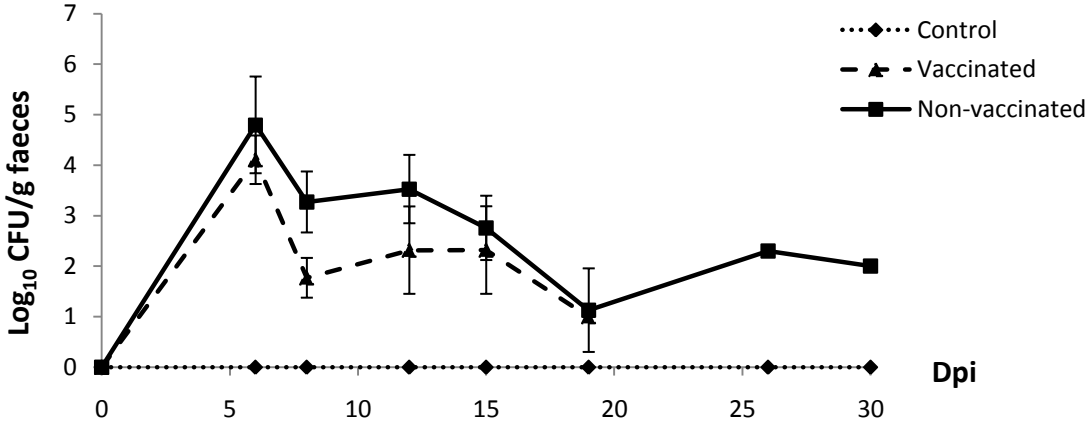
Data were analysed with SPSS17 using one-way ANOVA. A *p*-value < 0.05 was considered statistically significant.

3.4 Results

3.4.1 Excretion of *E. coli* O157:H7

All re-infected sheep excreted *E. coli* O157:H7 in the faeces (Figure 1a and b). Although excretion values of vaccinated sheep were lower than those of non-vaccinated sheep, these were not significantly different at any time ($p > 0.05$). Animals were euthanized when shedding decreased and became detectable only by IMS, suggesting a possible imminent clearance of *E. coli* O157:H7. The vaccinated sheep were positive by direct counts up to 15 dpi (8 - 15 days) and by IMS up to 19 dpi. They were euthanized at 22 dpi. Three out of four non-vaccinated sheep were positive by direct counts up to 15 dpi (12 –15 days) and by IMS up to 19 dpi (15 – 19 days). These three sheep were euthanized at 20 dpi, whereas the remaining non-vaccinated animal was positive by direct counts and IMS up to 30 dpi and was euthanized at 34 dpi (Figure 1b).

(a)



(b)

Animals	dpi	6	8	12	15	19	20	22	26	30	34	AUC
Vaccinated												
	4931	3.15	2.30	2.00	2.00	1.00[a]		†				30.0
	4948	4.62	1.00[a]	3.94	3.95	1. 00[a]		†				55.0
	4946	4.54	2.00	1.00a	1.00a	1. 00[a]		†				36.7
Non-vaccinated												
	4906	5.40	4.40	3.85	3.92	3.51			2.30	2.00	†	98.0
	4942	7.17	4.20	5.21	2.70	0.00[b]	†					69.0
	4893	3.83	2.00	2.00	1.00[a]	0.00[b]	†					31.8
	4897	2.78	2.48	3.04	3.40	1.00[a]	†					46.6

Figure 1: Excretion of *E. coli* O157:H7 by control, vaccinated and non-vaccinated animals. a) Average $\log_{10}$ values of CFU/g faeces of animals in a group ± SEM. b) Individual $\log_{10}$ values (CFU/g faeces). AUC: area under the curve, estimation of the total amount of shedding. *E. coli* O157:H7 was not recovered from control animals at any time. Dpi: days post inoculation. [a] positive only by IMS, [b] negative by IMS, † euthanasia.

3.4.2 Serum antibody responses and recirculating ASC

Serum IgG titers were determined by ELISA (data not shown). Vaccinated animals showed high titres (6.32 – 10.32) against all antigens used for the intramuscular immunizations. In contrast, the titres of the non-vaccinated animals were low and similar to the titres of the control animals (3.32 – 4.32).

To evaluate the presence of circulating ASC recognizing intimin, EspA, EspB or Tir, PBMC were isolated on 5 dpi (Table 3). No IgM ASC were detected as could be expected since the animals were re-infected and the vaccinated group had been hyper-immunized. The vaccinated animals showed IgG responses against intimin and EspA, likely reflecting the response after the intramuscular immunizations. However, there was no response against EspB and Tir. Three out of four non-vaccinated animals showed IgG responses against intimin and EspA, but lower than the response in the vaccinated animals. In contrast, IgA ASC specific for intimin and EspA were only detected in non-vaccinated animals. One non-vaccinated animal, 4897, was not responding at all.

Table 3: Number of antigen-specific antibody-secreting cells per 5 x 10^6 PBMC, isolated 5 days post inoculation from control and re-infected vaccinated and non-vaccinated sheep.

Group		*Intimin*		*EspA*		*EspB*		*Tir*	
	Animal	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
Control									
	507	0	0	0	0	0	0	0	0
	517	0	0	0	0	0	0	0	0
	578	0	0	2	0	0	0	0	0
	493	0	0	0	0	0	0	0	0
Vaccinated									
	4931	65	0	49	0	4	0	0	0
	4948	5	0	1	0	0	0	0	1
	4946	13	0	6	0	0	0	1	0
Non-vaccinated									
	4906	2	0	17	12	0	0	1	0
	4942	9	4	0	0	0	2	0	0
	4893	2	0	11	12	0	0	0	0
	4897	0	0	0	0	0	0	0	0

3.4.3 Lymphocyte proliferative responses

Proliferative responses are presented in Figure 2. The vaccinated animals did not seem to show proliferation towards three out of the four antigens used for vaccination as responses did not change from 0 to 19 dpi. A response was only observed after stimulation with EspB at day 0. It is not clear why there was no proliferative response towards the other antigens. Non-vaccinated animals seemed to respond to the infection at 19 dpi, as indicated by increased SI-values for intimin, EspA and Tir, although there were no significant differences ($p > 0.05$). Interestingly, shedding levels had dropped substantially by 19 dpi for most animals (Figure 1).

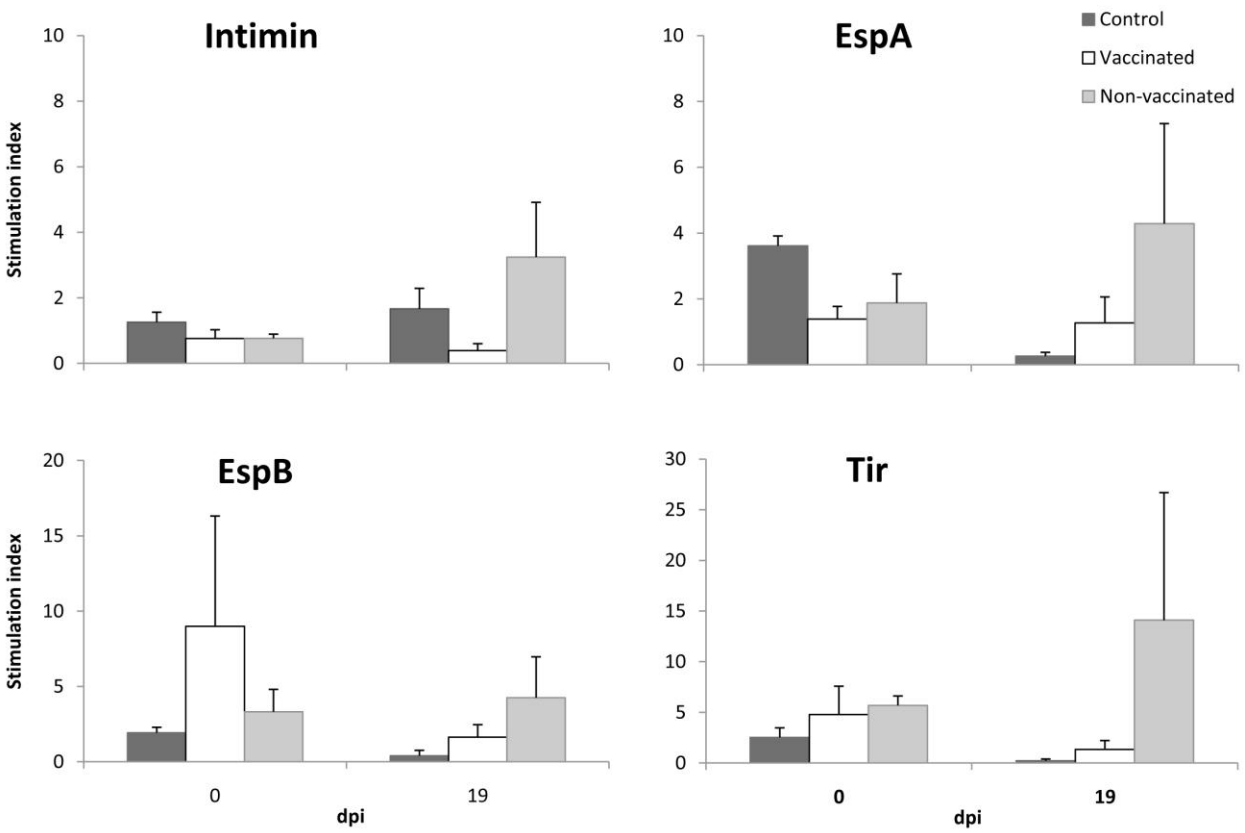


Figure 2: Lymphocyte proliferative responses of PBMC from control and re-infected vaccinated and non-vaccinated sheep, following *in vitro* stimulation with intimin, EspA, EspB and Tir (mean ± SEM). Dpi:days post inoculation.

3.4.4 *E. coli* O157:H7-specific ASC in spleen, lymph nodes and intestinal sites

To investigate the localization of the O157-specific antibody response when the decrease in shedding occurred, the number of intimin-, EspA-, EspB- and Tir-specific IgG and IgA ASC were determined in the spleen, MLN, CoLN, RLN, JPP, IPP and LPR (Figure 3a). Vaccinated animals had intimin- and EspA-specific IgG ASC in the spleen, as a result of the intramuscular immunizations. However, IgG responses against EspB and Tir were very low, and were in line with what was seen for PBMC at 5 dpi. IgG responses in the draining lymph nodes were low against all four antigens. Interestingly, the vaccinated animals had a clear EspA-specific IgG response in the JPP, in contrast to the non-vaccinated animals which showed only an EspA-specific IgG response in the IPP. In the rectal lamina propria, a very high background for IgG and IgA was observed in the control group against all four antigens, making interpretation impossible. IgA ASC were absent or present only in very low numbers in the other tissues.

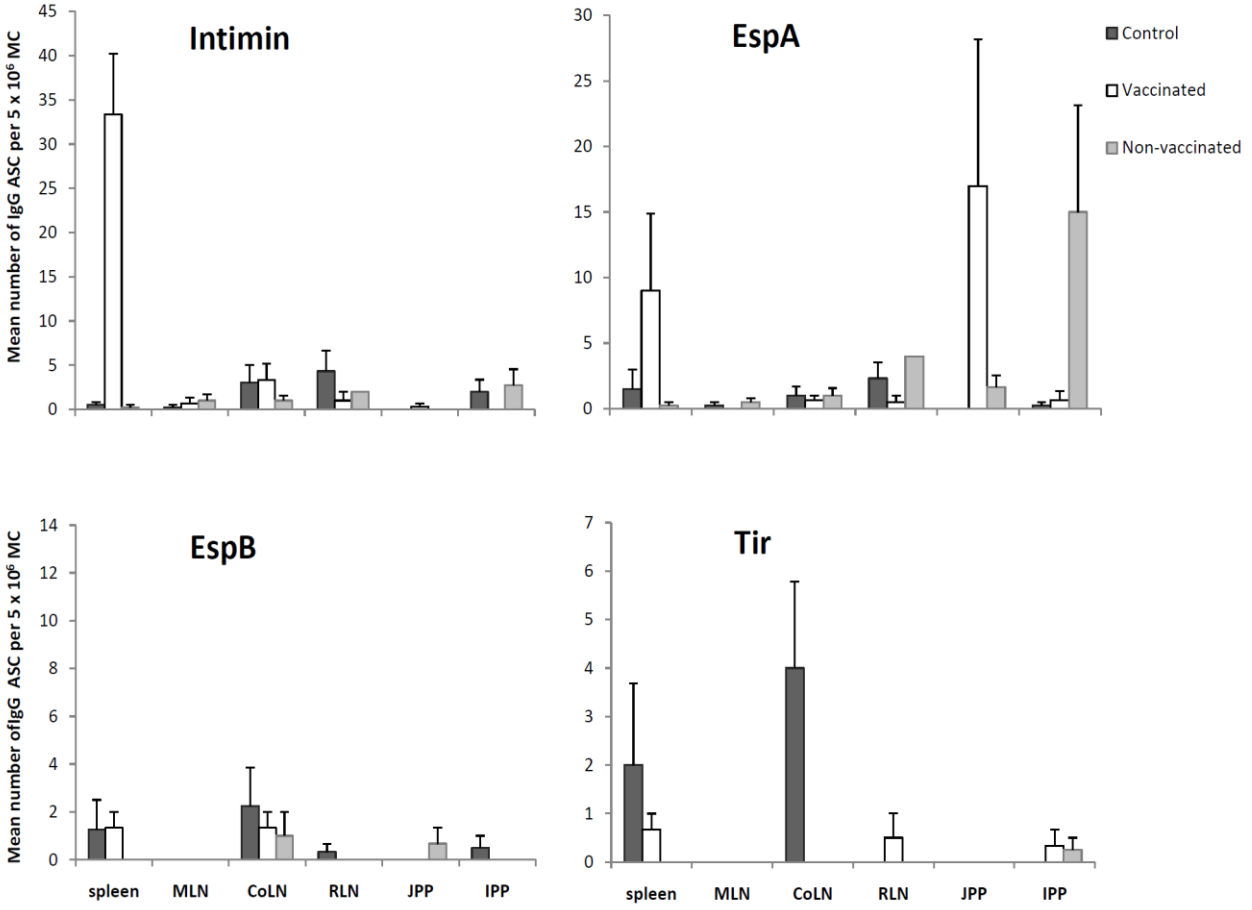


Figure 3: *E. coli* O157:H7-specific IgG ASC per 5 x 10^6 cells in various tissues in the control, vaccinated and non-vaccinated animals isolated at euthanasia (mean ± SEM). MLN: mesenteric lymph node (LN), CoLN: colon LN, RLN: rectal LN, JPP: jejunal Peyer's patches (PP), IPP: ileal PP.

3.4.5 Evaluation of cytokine responses of *in vitro* restimulated MC

To address the role of cytokines in clearance of *E. coli* O157:H7 infection, we analysed IL-4, IL-10, IL-12, IFN-γ and TNF-α production of *in vitro* restimulated MC from the different tissues. IL-4 was not detected in any of the samples. In general, the cytokine secretion patterns in the different groups of animals were similar regardless of the antigen that was used for stimulation. However, there were clear differences between some of the cytokines. IL-12 and IFN-γ secretion was very similar (Figure 4). Both cytokines were produced by spleen and lymph node cells in response to all four antigens, but were low to absent following stimulation of PP and LPR cells. IL-12 and IFN-γ responses of non-vaccinated animals were significantly higher in MLN cells compared to those of vaccinated animals. This was also the case for IL-10 production in the MLN. IL-10 and TNF-α were produced by every tested cell type, although secretion by PP and LPR cells was generally lower (Figure 5). Interestingly, TNF-α production seems to be suppressed in EHEC-infected animals compared to controls, whether they were vaccinated or not.

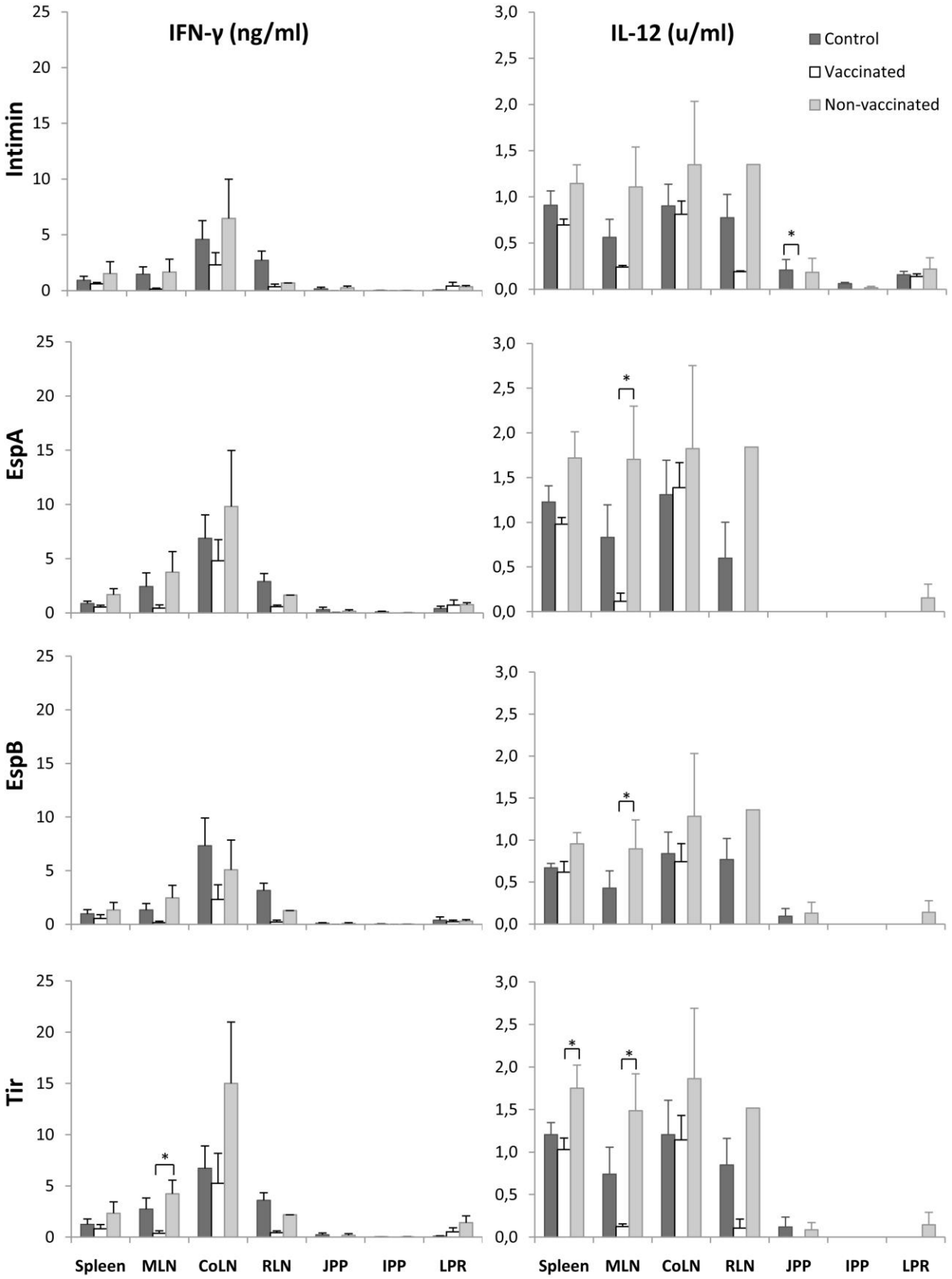


Figure 4: IFN-γ and IL-12 concentration in the supernatans of *in vitro* restimulated cells from various tissues isolated at euthanasia (mean ± SEM). MLN: mesenteric lymph node, CoLN: colon LN, RLN: rectal LN, JPP: jejunal Peyer's patches, IPP: ileal PP, LPR: rectal lamina propria. * significant difference ($p < 0.05$) between groups.

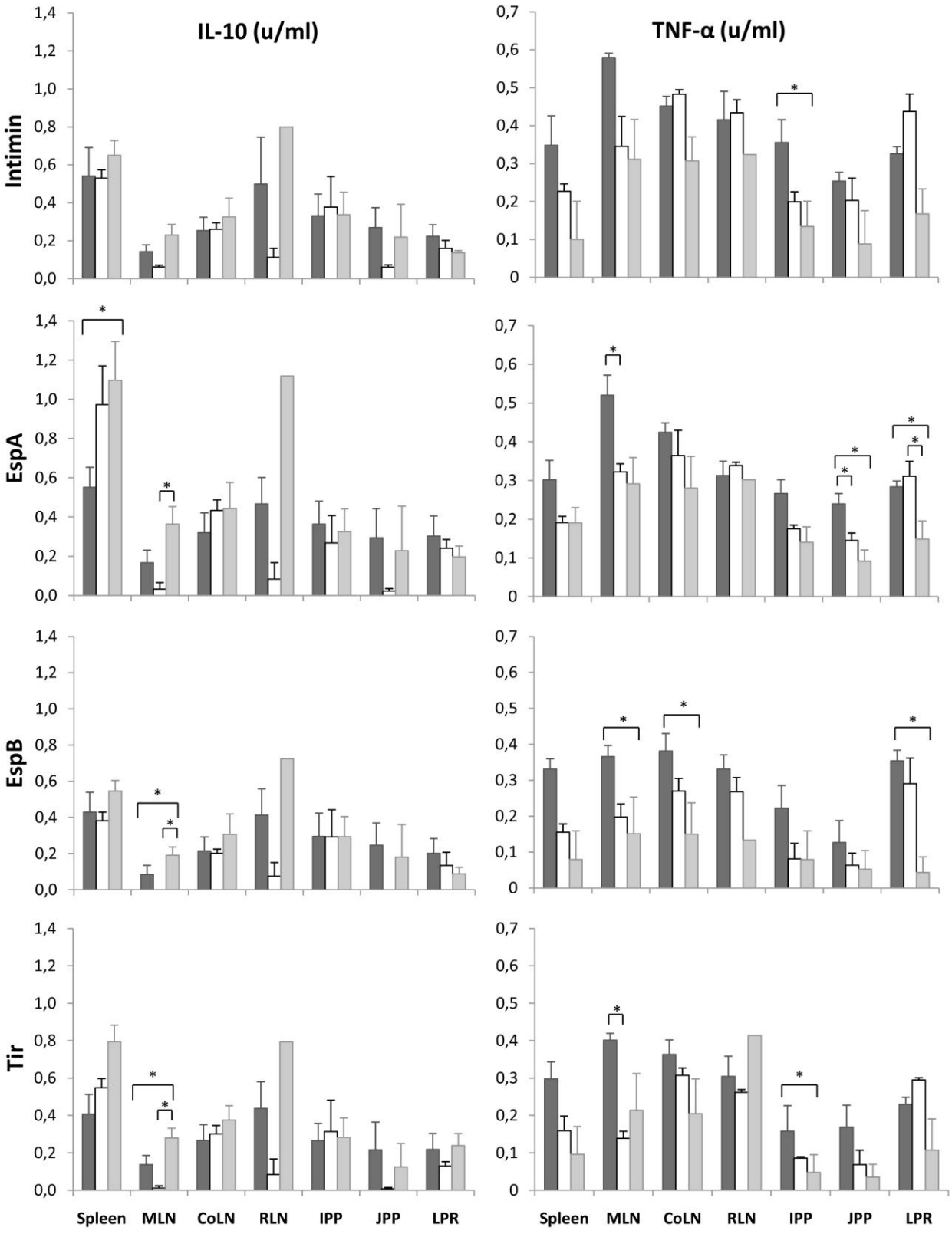


Figure 5: IL-10 and TNF-α concentration in the supernatans of *in vitro* restimulated cells from various tissues isolated at euthanasia (mean ± SEM). MLN: mesenteric lymph node, CoLN: colon LN, RLN: rectal LN, JPP: jejunal Peyer's patches, IPP: ileal PP, LPR: rectal lamina propria. * significant difference ($p < 0.05$) between groups.

3.5 Discussion

The role of the host immune response during *E. coli* O157:H7 infection is largely unknown. We have previously shown that *E. coli* O157:H7 evokes antibody and cellular responses during experimental infection of sheep. However, these immune responses do not protect sheep against re-infection (Vande Walle et al., 2010b). Since excretion of *E. coli* O157:H7 eventually declines and disappears after experimental inoculation, the present study was designed to gather information about the role of the immune response during clearance of infection. Previously infected animals (Atef Yekta et al., 2010) were re-infected and euthanized when shedding declined to determine the immune response at that time. Because vaccination with proteins of the type III secretion system appears to induce partial protection in cattle and sheep (Potter et al., 2004; Atef Yekta et al., 2010), we included hyperimmunized sheep that were also re-infected to evaluate the effect of pre-existing responses against intimin, EspA, EspB and Tir. The hyperimmunized animals had high serum antibody levels, antigen-specific circulating PBMC early after re-infection and a high number of antigen-specific antibody secreting cells in the spleen as a result of the systemic immunization. Curiously, proliferative responses were low to absent despite a booster immunization one week before the infection. In contrast, the non-vaccinated animals did not have serum antibodies but reacted to the infection with proliferative responses. More differences between the groups of animals were apparent in their cytokine responses, with regulatory and Th1 cytokines (IL-12, IFN-γ) being lower in the vaccinated animals compared to the non-vaccinated animals. This could indicate suppression of the cell-mediated immunity. Differences between vaccinated and non-vaccinated animals were also observed for the anti-inflammatory cytokine IL-10. Levels of IL-10 showed the tendency to be higher in spleen, mesenteric and rectal lymph nodes of non-vaccinated animals whereas production was lower in mesenteric and rectal lymph nodes, and jejunal Peyer's patches of the vaccinated animals. The results could suggest that infection with *E. coli* O157:H7 suppresses the inflammatory response by enhancing IL-10 production. Despite the distinct immune response, vaccination did not result in a significant reduction of shedding.

It is remarkable that re-infected non-vaccinated animals did not respond serologically to the infection, whereas this was the case in our previous study (Vande Walle et al., 2010b). However, in the latter study, animals were re-infected 66 days after the primary infection, whereas the period between the primary infection (Atef Yekta et al., 2010) and the re-infection described in the present study was twice as long (132 days). This may indicate that there is no

induction of long-lasting memory B-cells against the tested antigens. Nevertheless, IgG-and IgA-secreting cells recognizing intimin and EspA were observed among circulating PBMC 5 days after the re-infection in the present study. Whereas IgG-secreting cells were observed with both non-vaccinated and vaccinated animals, IgA-secreting cells were only present in non-vaccinated animals. This IgA response could be a reflection of what is happening in the intestine at that time. Isolating PBMC early after infection could be an interesting way to demonstrate a recent infection, as they probably respresent a circulating population of immune cells that were primed in the intestine. In the future, this method could be used to monitor PBMC responses during the course of infection.

Rectal and oral inoculation of sheep resulted in a different immune response, with serum antibodies only detected after oral inoculation (Vande Walle et al., 2010a; Vande Walle et al., 2010b). Therefore, we hypothesized that *E. coli* O157:H7 has to pass the small intestine to induce antibody responses. Peyer's patches are known to be important induction sites of immune responses, and indeed, antigen-specific antibody secreting cells were isolated from the Peyer's patches of the re-infected animals. Interestingly, vaccinated animals reacted only in the jejunal peyer's patches, in contrast with a response in the ileal Peyer's patches of the non-vaccinated animals. When individual data are considered, we noticed that animal 4906 in the non-vaccinated group did not respond in the ileal Peyer's patches (data not shown). Remarkably, this animal showed longer excretion than the others in that group. Moreover, animal 4906 had higher IL-10 and IL-12 levels than its group mates. We previously noted such differences between long- and short-term shedders (Vande Walle et al., 2010b). However, drawing conclusions from single observations is far too premature and these findings will have to be further investigated in a larger number of animals. Furthermore, evaluating more cytokines will provide a more complete profile of the immune response.

Very few reports of recurrent *E. coli* O157:H7 infections in humans have been reported (Robson et al., 1993; Siegler et al., 1993). Serum antibodies against LPS and Stx were reported in healthy farmers, suggesting acquisition of immunity to *E. coli* O157:H7 (Evans et al., 2000). Experimental *C. rodentium* infection in mice, a highly appreciated animal model for human *E. coli* O157:H7 disease, induces haemorrhagic colitis but protects mice against secondary challenge and results in development of long-lived humoral immune responses to intimin, EspA, EspB and Tir (Ghaem-Maghami et al., 2001; Mundy et al., 2005). In contrast, cattle and sheep do not develop disease and are susceptible to re-infection, both in experimental models and in natural

settings (Sanderson et al., 1999; Naylor et al., 2007; Vande Walle et al., 2010a; Vande Walle et al., 2010b). From our previous results and the results presented in this study, we conclude that there is no induction of a complete protective immunity against *E. coli* O157:H7 in these species, or that it is not sufficiently long-lasting to prevent subsequent infections. In contrast, it is known that intestinal clearance of *C. rodentium* in mice requires B-cells and IgG, but not secretory IgA (Maaser et al., 2004). Our results also indicate the dominance of IgG over IgA responses in *E. coli* O157:H7 infection in sheep, as IgA was barely detected among PBMC's early after infection or among intestinal ASC. IgG1 is prominent within mucosal secretions in sheep and cattle, suggesting that this isotype may play an important role in mucosal immunity (McNeilly 2008). Nevertheless, mucosal IgA responses have been demonstrated in cattle following *E. coli* O157:H7 infection (Bretschneider et al., 2008; Nart et al., 2008a). Still, the present study, however preliminary, indicates an important role of mucosal immune responses during *E. coli* O157:H7 infection.

3.6 Acknowledgements

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Part IV: General discussion and future perspectives

Introduction

Since its identification in 1982, *E. coli* O157:H7 has been the subject of many research projects. An important part of the work focused on the development of reliable and sensitive detection methods, to diagnose infections, to determine the prevalence and to identify the public health risk. Gradually, more information became available regarding the reservoir and the transmission routes of infection. Although being the causative agent of serious illnesses and even death in humans, *E. coli* O157:H7 resides apparently quietly in ruminants. Ruminants lack the Gb3 receptor on endothelial cells and are therefore not susceptible to the effects of Stx, the cause of bloody diarrhoea and HUS in humans. The bacterium colonizes the gastrointestinal tract of ruminants and has developed means to persist in animals for long periods of time without causing clinical signs. But how is it possible that *E. coli* O157:H7 can persist without being eliminated by the immune system? It has been hypothesized that the bacterium suppresses the immune system of the host. However, not much is known regarding the interaction of the bacteria with the immune system and therefore the aims of this thesis were to determine the immune response against *E. coli* O157:H7 and to obtain insights in the role of the immune system in persistence of *E. coli* O157:H7 in ruminants.

***E. coli* O157:H7 elicits non-protective immune responses**

To study the interaction of *E. coli* O157:H7 with the immune system of ruminants, we developed two sheep infection models. In a first model, sheep were inoculated with *E. coli* O157:H7 via a rectal route (Chapter 1). The choice of this route was based on the identification of the terminal rectum as a primary colonization site in cattle (Naylor et al., 2003). Following rectal inoculation of cattle, *E. coli* O157:H7 adhered to the rectal mucosa, leading to persistent colonisation and long-term recovery of the bacteria in the faeces (Sheng et al., 2004). In our study, rectal inoculation of sheep with a high dose of *E. coli* O157:H7 led to shedding of the bacteria for several weeks and even months in sheep inoculated by a method referred to as the 2-days-sponge method. The immune system of the host was able to recognize the bacterium, reflected by a cellular immune response against intimin, EspA and EspB, three important virulence factors of *E. coli* O157:H7. However, the presence of this response did not prevent animals from becoming colonized after a second inoculation. Surprisingly, an antibody response could not be demonstrated. Nevertheless, antibodies have been detected in naturally infected sheep (Vande Walle et al., unpublished results) and cattle (Cristancho et al., 2008; Vilte et al., 2008, Joris et al., in preparation). We investigated this discrepancy in a second study where an

oral infection model was used, mimicking the natural route of infection (Chapter 2). In that study, both cellular and antibody responses were demonstrated which followed the course of infection: rising after the inoculation and decreasing as shedding decreased. When animals were re-inoculated, responses rose again and the antibody response sustained even when shedding had disappeared. However, this response was not protective, as demonstrated by the fact that animals could become re-colonized following a second experimental inoculation. This indicated that shedding can re-occur as seen in the field, where animals become infected by much lower doses and shedding is typically intermittent. Even after several consecutive negative testings, shedding can re-occur (Ellis-Iversen et al., 2009). Re-infection could arise from re-ingestion from the environment although it is also possible that *E. coli* O157:H7 is not cleared from the gastrointestinal tract, but remains present in very low numbers, too low to be detected even by highly sensitive methods, ready to re-colonize the intestine. Moreover, detection of *E. coli* O157:H7 in faecal samples might be an underestimation of its true prevalence. The bacterium might reside in a particular intestinal location different from the rectum while temporarily not being excreted. This hypothesis is supported by a recent study in naturally infected cattle, where *E. coli* O157:H7 was found throughout the gastrointestinal tract and presence of *E. coli* O157:H7 in any intestinal location could not predict the presence in faecal or recto-anal junction samples. Interestingly, an almost 100% correlation was found between recovery of *E. coli* O157:H7 from intestinal content and mucosal samples (Keen et al., 2010), indicating mucosal colonization at multiple sites.

While persisting in such low numbers, *E. coli* O157:H7 might be invisible for the immune system. Possibly *E. coli* O157:H7 can remain in the intestine, seemingly undetectable, until it becomes reactivated by environmental changes such as changes in diet, environmental temperature, or through ingestion of more *E. coli* O157:H7 from the environment or modifications in the intestinal flora. Such changes could explain the raised prevalence of *E. coli* O157:H7 during the warmer summer months, when animals are moved from the stable to pasture. This movement also includes a change in diet and in physiological health status of the animals. Encountering more species congeners could trigger the mechanisms of quorum sensing, and enhance bacterial replication. When the bacteria are present in sufficient numbers, the immune system could become activated, leading to suppression of the bacteria once more. Until the next change occurs. This could account for the typically intermittent shedding pattern that is observed. An important question is why an individual animal would shed for a longer period of

time than another animal of the same breed and age, that has been exposed to the same strain and dose in similar experimental conditions. In our studies we identified long and short time shedders (Chapter 2 and 3). We hypothesized that the host immune response could play a role. Indeed, the data, although preliminary and from a limited number of animals, point to a differentially regulated immune response. This should be further investigated. A limitation is the accessibility of intestinal tissues, which are generally sampled at euthanasia. We hypothesized that interesting information on immune mechanisms could be obtained at cessation of shedding. However, the procedures to monitor shedding are time-consuming, the exact moment of cessation cannot be predicted and therefore it is difficult to determine a good timing for euthanasia. Cannulation of the draining lymph nodes allows to keep the animals alive, and to monitor the shedding and immune response throughout the course of infection (Scheerlinck et al., 2008). Flow cytometry can be used to identify the type of immune cells involved in this response. Before, these studies were hampered by the lack of suitable reagents, whereas recently many efforts have been made to develop ovine markers (Entrican et al., 2009).

Cytokines modulate immune cell functions and exert important roles during infection (Scheerlinck and Yen, 2005). In this thesis, we studied the production of different classes of cytokines (Chapter 3). and IL-4 represent Th1 and Th2 cytokines, respectively. TNF-α is a pro-inflammatory cytokine, whereas IL-10 is considered anti-inflammatory and needed to downregulate IFN-γ production. IL-12 on the other hand, stimulates IFN-γ production. We have observed differences in cytokine production between vaccinated and non-vaccinated animals, with indications that *E. coli* O157:H7 suppresses the cell-mediated immunity. However, we should be careful to draw conclusions from this preliminary study. Studying more cytokines could provide a more complete profile of the immune response. A subclass of immune cells worth investigating are the Th17 cells. They have recently been identified as a new class of CD4+ helper T-cells in addition to Th1, Th2, Th3 and Treg cells and are characterised by the production of IL-17. Th17 cells play an important role in the defence against extracellular pathogens, particularly Gram negative bacteria that colonize mucosal surfaces (Peck and Mellins, 2010). Furthermore, they have been implicated in *C. rodentium* infection. Infection with *C. rodentium* in mice did not strongly influence the expression of Th1 or Th2 cytokines, but affected the expression of Th17 cytokines, with significant upregulation of IL-17 and IL-6 at the peak of infection (8-10 dpi) and during clearance (21 dpi) (Symonds et al., 2009). IL-22, another cytokine

produced by Th17 cells, is required for the early host defense against *C. rodentium* (Zheng et al., 2008). Th17 responses to EHEC infection have not been investigated to date.

Mucosal immunity

One of the most important findings of this thesis was the difference in gastrointestinal localization of *E. coli* O157:H7 following experimental infection, and the implication of this localization for the presence of immune responses. The bacteria were found exclusively in the lower gastrointestinal tract of sheep inoculated rectally (Chapter 1), whereas oral inoculation led to recovery of *E. coli* O157:H7 from sites throughout the entire digestive tract (Chapter 2). In addition, the observation that serum antibody responses were only detected after oral inoculation but not after rectal inoculation, suggests that *E. coli* O157:H7 has to pass the small intestine to evoke antibody responses. Cellular responses were present after both rectal and oral inoculation, indicating that these responses can be initiated in the lower gastrointestinal tract. The ovine rectal mucosa is known to contain lymphoid follicles, similar to Peyer's patches in the small intestine which are induction sites of immune responses (Sedgmen et al., 2002). Indeed, antigen-specific antibody-secreting cells were detected in the rectal lamina propria (Chapter 2). This response was the strongest in an animal that had very recently stopped shedding, whereas it was virtually absent in another animal that ceased shedding almost 6 weeks earlier. In addition to the response found in the rectal lamina propria, antigen-specific antibody secreting cells were found in the jejunal and ileal Peyer's patches of re-infected animals (Chapter 3). Interestingly, such a response was not detected in one animal which was still shedding at the time of euthanasia and was excreting for a longer period of time than the other animals in its group. These findings indicate that mucosal immune responses are important during *E. coli* O157:H7 infection, but mucosal protection is possibly limited in space and time.

Why are the immune responses not protective?

Although all vaccination protocols tested to date rendered serum antibodies and even mucosal antibodies against the target proteins, some were ineffective and none were able to completely reduce faecal shedding of *E. coli* O157:H7 (Potter et al., 2004; McNeilly et al., 2008; Thornton et al., 2009). Generally a reduction in the amount of shed bacteria was achieved, or vaccinated cattle were less likely to shed *E. coli* O157:H7 than non-vaccinated cattle (Peterson et al., 2007b; Thornton et al., 2009). However, even when fewer cattle were shedding, the ones that did, continued to shed in high amounts (McNeilly et al., 2008).

Increasing amounts of evidence point to an immunomodulatory effect of EHEC. Early inflammatory responses are present, even before colonisation has been established, but do not offer protection against infection. It is likely that the inflammatory response is dampened by EHEC effectors. For instance, EHEC negatively modulates host signalling cascades like the IFN-γ-mediated signal transduction pathway (Ceponis et al., 2003; Jandu et al., 2007). In our studies it was clear that EHEC infection induces immune responses that are not protective. Moreover we did not see signs of suppression as cellular responses were still present and lymphocytes responded well to mitogen stimulation with ConA (unpublished results). Recently, an immunomodulatory role of the related pathogen EPEC has been shown, whereby the wild type strain induced massive influx of neutrophils and macrophages and secretion of proinflammatory cytokines in the ileum of orally inoculated rabbits without recruitment of T-cells or T-cell induced cytokines. A *tir/espB* deletion mutant did not exert these effects, demonstrating the role of injected effector molecules (LeFoll et al., 2010). To confirm these results for EHEC infection in sheep, the infiltration of immune cells in different tissues should be investigated, preferably at different time points after infection.

***E. coli* O157:H7: an invincible pathogen?**

E. coli O157:H7 gains its strength from 1) its great survival capacity to enter the saliva barrier in the oral cavity and resist the acidic environment of the stomach, thereby entering the small and finally the large intestine, 2) its remarkable adhesion mechanism, resulting in a strong intimate adhesion to the host cell, 3) its ability to withstand host immune responses. During evolution, *E. coli* O157:H7 has acquired several virulence factors enabling these mechanisms, each time gaining in virulence capacity. By means of its effector molecules, it seems that EHEC can counteract every possible attack of the host. Increased mucus secretion to flush away loosely adherent bacteria is tackled by the action of the mucinase StcE (Grys et al., 2006; Bergstrom et al., 2010), whereas Cif blocks the cell cycle of infected epithelial cells, thereby preventing cell death and subsequent elimination of the pathogen. Although the development of immune responses is not arrested as shown in this thesis, the action of immune cells is countered by lymphostatin, which blocks the proliferation of lymphocytes (Abu-Median et al., 2006). Furthermore, the innate immunity is subverted by resisting the complement pathway through the effects of Stx2, StcE and EspP (Lathem et al., 2004; Orth et al., 2009; Orth et al., 2010). EspP has recently been shown to specifically cleave C3b and C5 of the complement pathway (Orth et al., 2010). This finding may explain how *E. coli* O157:H7 can resist the antibody

response. The complement pathways are activated by antigen-antibody complexes and other structures on the pathogen surface and lead to phagocyte recruitment, opsonisation and lysis by the membrane attack complex. C3b and C5 are involved in many of these processes, and are rendered inactive by EspP. Furthermore, the action of the recently discovered subtilase cytotoxin cleaves secreted antibodies (Hu et al., 2009). In the light of these recent findings, it becomes more and more clear that *E. coli* O157:H7 does not inhibit the early development of immune responses. However, the pathogen has acquired multiple ways to resist the effector functions of the immune response, allowing *E. coli* O157:H7 to persist in the presence of an adaptive immune response. Thus, EHEC can multiply and colonize for a long time. The factors leading to its ultimate removal are yet unknown.

Role of Shiga toxin in infection

Immune responses to intimin, EspA, EspB and in the third study also Tir were examined in this thesis. All four molecules are important colonization factors, mediating adherence of *E. coli* O157:H7 to the epithelium. Data from experimental studies have shown that intimin and Tir are necessary for persistence (Sheng et al., 2006b). Nevertheless, other molecules like H7 or OmpA play a role in the adhesion and pathogenesis and are worth to be studied. In the first place, the influence of Shiga toxin on immune responses should be investigated. We used a Shiga toxin-negative strain, which was used before in several *in vitro* and *in vivo* infection models and can persist in sheep (Dibb-Fuller et al., 2001; Wales et al., 2002; Woodward et al., 2003, our studies). The role of Stx in colonisation is unclear. In cattle, Shiga toxins are likely degraded before they can influence the immune response (Hoey et al., 2003) and they are also not involved in *E. coli* O157:H7 colonisation of the lymphoid follicle-associated epithelium in the terminal rectum (Sheng et al., 2006b). It was reported that Stx2 does not play a role in the colonization of *E. coli* O157:H7 in sheep (Cornick et al., 2007), whereas it enhanced adhesion of *E. coli* O157:H7 to cattle intestinal tissue explants (Lowe et al., 2009). However, whereas the first study used isogenic strains to investigate the role of Stx, the latter study determined colonization of strains of different lineages. Enhanced colonization of Stx2-positive *E. coli* O157:H7 compared to the isogenic deletion mutant in mice was attributed to increased *in vitro* expression of nucleolin by Stx2 (Robinson et al., 2006), although the significance of this receptor in colonization is unclear (Sinclair et al., 2006). *In vitro* studies have demonstrated that Stx is capable of suppressing the proliferation of bovine lymphocytes. The influence of Stx on development of immune responses against other virulence factors should be looked into. Experimental infections with Stx positive

strains require specialized animal facilities and should be carefully conducted to avoid human infections and spread of the strain into the environment.

Differences between human and ruminant hosts

E. coli O157:H7 is generally regarded as a harmless bacterium in cattle. Nevertheless, Baines et al. (2008) identified mucosal haemorrhages in experimentally inoculated cattle and stated that the bacterium is in fact a ruminant pathogen. Since long a differentially regulated effect of *E. coli* O157:H7 on human and bovine cells has been suspected, but it should be formally investigated whether this is the case. It has been shown that ruminants are not susceptible to Stx-induced disease, but this does not mean they are not affected by other pathogenic mechanisms. To investigate the difference between human and ruminant infection, it would be interesting to further study cellular responses, e.g. by studying the effect of *in vitro* stimulation with *E. coli* O157:H7 proteins on human, bovine and ovine lymphocyte proliferation and cytokine production.

The evidence supporting the hypothesis of immune suppression in ruminants is mainly derived from *in vitro* experiments, whereas monitoring the full course of an immune response in humans as can be done in ruminant experimental infection models is impossible due to obvious ethical objections. Immune responses against different *E. coli* O157:H7 molecules, such as intimin, EspA, EspB, LPS, H7 and Stx have been identified in both humans and ruminants. Nevertheless, the full repertoire of immunogenic determinants of *E. coli* O157:H7 is not known. A better knowledge of these antigens might be crucial in understanding the difference between the human and ruminant host and may aid in designing an effective control measure for the spread of *E. coli* O157:H7. Approaches such as protein micro-arrays have been successful in identifying antigens that are differentially recognized between humans and animal reservoir hosts of the zoonotic pathogen *Brucella melitensis* (Liang et al., 2010).

Theories in the margin

E. coli O157:H7 persists inside the ruminant reservoir host apparently without causing harm and is spread into the environment, from where it is eventually transmitted to humans. It was shown that this is not a merely commensal relationship: *E. coli* O157:H7 causes a certain amount of damage to the ruminant's intestinal tract in the form of focal mucosal haemorrhages associated with *E. coli* O157:H7 colonisation sites (Baines et al., 2008). Then what is the advantage for the ruminant to harbour these bacteria? A possible explanation is that the

presence of STEC renders cattle and sheep less susceptible to viruses, such as bovine leukemia virus, bovine immunodeficiency virus and equine infectious anaemia virus. Animals that carried high numbers of STEC remained in good health, whereas low numbers were correlated with a poor prognosis in sheep infected with bovine leukemia virus (Ferens et al., 2008). Possibly STEC influences IFN-γ en IL-12-dependent pathways, which are correlated with resistance to bovine leukemia virus (Kabeya et al., 2001). As with *E. coli* O157:H7, many parasitic infections such as *Cooperia* in calves result in distinct shedding patterns (Kanobana et al., 2003). Animals can be classified into low, intermediate and high responders, correlated to the speed by which worms are expelled from the host due to an immune response (van Diemen et al., 1997; Kanobana et al., 2001). IgE is known to play a role in immunity against parasitic infections, so it could be worthwhile to investigate the role of IgE in *E. coli* O157:H7 infections in ruminants.

Many pathogenic micro-organisms that cause chronic infections undergo mutations to withstand the immune system, the best example being the human immunodeficiency virus which continuously changes its virulence determinants. In particular, outer-membrane determinants are changed under immunological pressure. *E. coli* O157:H7 is prone to evolutionary changes, as evidenced by its origination from an EPEC O55 strain, by acquisition of the Shiga toxin and other virulence genes through horizontal transfer. Variants have been identified in important colonization factors such as intimin, EspA, EspB, Tir and smaller nucleotide differences between strains have been demonstrated. Moreover, certain strains are associated with elevated virulence (Laing et al., 2009), making it likely that a single *E. coli* O157:H7 strain can evolve and adapts itself to the environment, possibly under the pressure of the host's immune system. Usually different strains isolated from different sources are investigated, but to our knowledge the evolution of a single strain in time has not been investigated yet. A limited number of pulsed-field gel elektrophoresis (PFGE)-profiles could be identified among different strains isolated from farm animals, indicating low sequence diversity (Vali et al., 2005). Interestingly, there was a change in PFGE and phage types from isolates shed by cattle at pasture compared with those shed by the same cattle when weaned and housed in pens. In contrast, characterizations of *E. coli* O157:H7 isolates from Belgian farm cattle using PFGE, revealed that on each positive farm only one genotype was present, even after a period of more than six months (Cobbaut et al., 2008), suggesting there is very little selective pressure on *E. coli* O157:H7. More detailed investigations have shown positive selection pressure on *rhs* genes, which encode extracellular proteins that are possibly involved in promotion of survival of

E. coli O157:H7 during the intestinal transit (Liu et al., 2009), and a short segment in the central region of the *fliC* gene encoding flagellin has been under positive selection in the divergence of H6 and H7 alleles (Reid et al., 1999). Perhaps a far-fetched hypothesis, but positive selection might pose a mechanism for *E. coli* O157:H7 to resist the ruminant host's immune system.

Future perspectives

Despite the expanding knowledge regarding the colonization mechanisms of *E. coli* O157:H7, the research regarding the immune response to this pathogen is still in its infancy. The issues discussed above have highlighted important questions that should be addressed. Future work should focus on elucidating the role of Stx on the immune response, by comparing responses to Stx-negative and -positive strains. Simultaneously, efforts should be made to address the role of other antigens, to unravel the impact and the importance of these antigens during infection. This thesis and other research focused on intimin, EspA, EspB and Tir, key virulence factors during colonisation, however, it remains uncertain which antigens are defining the immune response to *E. coli* O157:H7. Techniques such as protein micro-arrays would be very helpful to identify immunogenic antigens and differences in immune responses between natural and carrier hosts, or between infected and non-infected animals (Liang et al., 2010). Furthermore, the immune cells taking part in the immune response should be identified. Cannulation and flow cytometry experiments are excellent ways to characterize the cell populations which determine the outcome of infection (Scheerlinck et al., 2008; Entrican et al., 2009). Finally, monitoring immune responses at different time points during infection will provide valuable information regarding infection kinetics, and may aid to answer the question why individual animals are prone to long-term shedding whereas others are not. This knowledge can be implemented in the design of efficient vaccines against *E. coli* O157:H7.

In conclusion, knowledge regarding the pathogenesis and persistence of EHEC and in particular serotype *E. coli* O157:H7 in ruminants still has many frontiers. Many *in vitro* data should be confirmed *in vivo*. Until a good vaccination strategy is found, hygienic measurements and precaution are the best way to avoid contamination with this pathogen.

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Summary

Summary

Enterohaemorraghic *E. coli* (EHEC) form a subgroup of Shiga-toxin producing *E. coli* serotypes closely associated with haemorraghic colitis (bloody diarrhoea) and the haemolytic uremic syndrome in man. Most cases are caused by infection with *E. coli* O157:H7. *E. coli* O157:H7 colonizes the gastrointestinal tract of ruminants and is excreted in the faeces. Ruminants constitute the major reservoir, spreading the infection to humans by a faecal-oral route. The animals do not suffer from infection as they lack the receptor for Shiga toxin in the intestinal epithelium. During slaughter the bacteria can be transferred to the carcass, posing a risk for foodborne infection when the meat is insufficiently heated prior to consumption. Other products at risk to become contaminated with *E. coli* O157:H7 are raw milk, cheese and vegetable crops. Consumption of EHEC-contamined food poses an important public health risk and has important economic consequences.

E. coli O157:H7 uses a type III secretion system to establish close contact with the host cell. Important colonization factors are intimin, EspA, EspB and Tir. Following adhesion, several effector molecules are injected into the host cell, which subvert the normal cellular processes. Although a great deal is already known regarding the colonization mechanisms of this pathogen in the intestine, it remains to be elucidated how and why *E. coli* O157:H7 can be persistently present in ruminants. It has been hypothesized that the immune system of the host is suppressed. To date, only limited information is available regarding the interaction of *E. coli* O157:H7 with the immune system of ruminants. The study of this interaction can contribute to a better understanding of the persistence of *E. coli* O157:H7 and may have applications in the reduction of the spread of these bacteria.

Part I of this thesis provides an overview of the literature. The pathogenesis in humans and the principal characteristics of the animal reservoir are described, as well as the current control and reduction strategies. Subsequently, the virulence factors of *E. coli* O157:H7 are discussed, followed by the effector molecules known to date. Finally, the immune response of humans and cattle against this bacterium is described.

Knowledge regarding the immune response of sheep is non-existent so far and therefore the general aim of this thesis was to gain insight in the interaction of *E. coli* O157:H7 with the immune system of sheep. Following questions were addressed in the experimental work of this thesis:

Does experimental infection, by a rectal or an oral route, lead to persistent colonization?
Does infection result in an immune response and does this protect against re-infection?
What is the role of this immune response in clearance of the infection in sheep?

To investigate the effects of colonization on the immune system of sheep, an infection model is needed that mimics a long-term infection as it can occur in both sheep and cattle. Since the terminal rectum has been identified as a primary colonization site in cattle, a rectal inoculation model was developed in the first study **(Chapter 1)**. Rectal application of a sponge, containing 10^{10} CFU *E. coli* O157:H7, administered on 2 consecutive days, resulted in shedding that endured 48 to 78 days. This method was used to analyse the immune response of infected sheep against virulence factors of *E. coli* O157:H7. The primary infection did not lead to a protective immune response, as demonstrated by the fact that the sheep became recolonized after the administration of a secondary inoculation. Although a cellular response occurred against intimin, EspA and EspB, surprisingly a serum antibody response was absent. Colonization of *E. coli* O157:H7 extended from the rectum to the distal large intestine, in contrast to the restricted colonization of the recto-anal junction observed in cattle.

Since serum antibodies are found in naturally infected sheep that most likely have ingested the bacteria, an oral infection model was used to infect sheep with *E. coli* O157:H7 in the second study **(Chapter 2)**. A primary oral inoculation resulted in shedding of *E. coli* O157:H7 in the faeces and detection of antibody responses against intimin, EspA and EspB. The antibody titres waned as shedding decreased. A secondary inoculation resulted in longer shedding, even though a booster antibody response occurred. Cellular responses followed a similar pattern as the antibody levels, although the cellular secondary response was lower. The presence of antigen-specific antibody-secreting cells indicates involvement of both a systemic response in the spleen and a local immune response in the terminal rectum. Combined with the results from the rectal inoculation study, these results imply that *E. coli* O157:H7 has to pass the small intestine to evoke antibody responses. Yet, the presence of antibodies does not protect against re-infection.

Shedding of *E. coli* O157:H7 by ruminants can last for weeks, even months, but eventually the infection ceases. To determine the role of the immune response in clearance of *E. coli* O157:H7, the third study was designed **(Chapter 3)**. Previously infected animals were re-infected with *E. coli* O157:H7 and euthanized when excretion decreased to analyse systemic and local immune responses to intimin, EspA, EspB and Tir. In addition, we investigated the influence of preceding immunization with these antigens. ELISPOT tests revealed mucosal antibody responses against EspA, in the jejunal Peyer's patches of vaccinated animals and in the ileal Peyer's patches of non-vaccinated animals. Cytokines IL-12 and IFN-γ were produced by mesenteric lymph node cells, whereas IL-10 and TNF-α were also produced by intestinal lymphocytes. Individual differences between longer and shorter shedding animals were observed, indicating the importance of mucosal immune responses during *E. coli* O157:H7 infection. However, *E. coli* O157:H7 infection does not seem to result in the induction of long-lasting memory cells, and mucosal protection might be limited in space and time.

The final part of this thesis contains the general discussion and main conclusions of this work. The experiments show that *E. coli* O157:H7 can colonize sheep for a long period of time. Passage through the small intestine appears to be important for the initiation of immune responses, as serum antibodies were only observed following oral and not following rectal inoculation. However, cellular responses were present in both cases. Although it was shown that cytokine responses occur when the infection pressure diminishes, the role of the immune system in clearance of the infection remains unclear. Further research is needed to elucidate the mechanism behind persistence of *E. coli* O157:H7 in cattle and sheep.

Samenvatting

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Enterohaemorraghische *E. coli* (EHEC) omvat een subgroep van Shiga-toxine producerende *E. coli* serotypes die nauw geassocieerd worden met haemorraghische colitis (bloederige diarree) en het haemolytisch uremisch syndroom bij de mens. De meeste gevallen worden veroorzaakt door infectie met *E. coli* O157:H7. *E. coli* O157:H7 koloniseert de darm van herkauwers en wordt uitgescheiden in de faeces. Herkauwers vormen het voornaamste reservoir, waarbij de infectie naar de mens verspreid wordt via een faeco-orale route. De dieren ondervinden zelf geen klinische gevolgen van de infectie omdat ze de receptor voor het Shiga toxine missen op het darmepitheel. Tijdens het slachtproces kan de bacterie op de karkassen terecht komen, wat kan leiden tot een voedselinfectie wanneer het vlees niet voldoende verhit wordt vóór consumptie. Andere risicoproducten zijn producten die mogelijks in contact zijn gekomen met besmette faeces zoals rauwe melk, kaas en groenten. Consumptie van met EHEC besmette voedingswaren vormt een belangrijk gezondheidsrisico voor de mens en heeft bovendien belangrijke economische gevolgen.

E. coli O157:H7 gebruikt een type III secretiesysteem om nauw contact te maken met de gastheercel. Belangrijke eiwitten in dit proces zijn intimine, EspA, EspB en Tir. Na adhesie worden verscheidene effectormoleculen in de gastheercel geïnjecteerd, die de normale processen in de cel verstoren. Hoewel reeds veel geweten is over de manier waarop deze pathogeen de darmen koloniseert, moet nog opgehelderd worden hoe en waarom *E. coli* O157:H7 persistent aanwezig kan blijven bij herkauwers. Mogelijks speelt een onderdrukking van het immuunsysteem van de gastheer een rol. Tot nu toe is er slechts beperkte informatie over de interactie van *E. coli* O157:H7 met het immuunsysteem van herkauwers. Het bestuderen van deze interactie kan bijdragen tot het begrijpen van de persistentie van *E. coli* O157:H7 en toepassing hebben in het reduceren van de verspreiding van deze bacterie.

Deel I van deze thesis geeft een overzicht van de literatuur. De pathogenese bij de mens en de voornaamste kenmerken van het herkauwer reservoir worden weergegeven, evenals een actueel overzicht van mogelijke bestrijdings- en inperkingsmiddelen. Vervolgens komen de virulentiefactoren van *E. coli* O157:H7 aan bod, evenals de tot nu toe gekende effectormoleculen. Tenslotte wordt de immuunrespons van de mens en runderen tegen deze bacterie beschreven.

Over de immuunrespons van schapen is echter tot nu toe niets geweten en het algemene doel van deze thesis was dan ook inzicht te krijgen in de interactie van *E. coli* O157:H7 met het immuunsysteem van schapen. Hoofdstukken 1 tot 3 geven het experimenteel werk van deze thesis weer. Daarbij werd een antwoord gezocht op volgende vragen:

Leidt experimentele infectie van schapen, hetzij via een rectale route, hetzij via een orale weg, tot een persistente kolonisatie?

Wordt er een immuunrespons opgebouwd en beschermt deze tegen herinfectie?

Wat is de rol van deze immuunrespons tijdens het verdwijnen van de infectie bij schapen?

Om het effect van kolonisatie op het immuunsysteem van schapen te bestuderen, is een infectiemodel nodig dat de langdurige infectie zoals die kan voorkomen bij schapen en runderen nabootst. Aangezien het terminale rectum werd geïdentificeerd als een primaire kolonisatieplaats bij het rund, werd een rectaal infectiemodel ontwikkeld in de eerste studie **(Hoofdstuk 1)**. Rectaal aanbrengen van een spons die 10^{10} bacteriën bevatte, en dit op 2 opeenvolgende dagen, gaf een uitscheiding die 48 tot 78 dagen aanhield. Deze methode werd gebruikt om de immuunrespons van geïnfecteerde schapen tegen virulentiefactoren van *E. coli* O157:H7 te analyseren. De primaire infectie leidde niet tot opbouw van een beschermende immuunrespons, wat bleek uit het feit dat de schapen opnieuw konden gekoloniseerd worden na toedienen van een secundaire inoculatie. Hoewel er een cellulaire immuunrespons optrad tegen intimine, EspA en EspB, was een serum antistoffenrespons verrassend genoeg afwezig. *E. coli* O157:H7 koloniseerde de darm van het rectum tot de distale dikke darm, in tegenstelling tot de beperktere kolonisatie van de recto-anale junctie bij het rund.

Omdat serum antistoffen aangetroffen worden bij natuurlijk geïnfecteerde schapen, die hoogstwaarschijnlijk de bacterie via de mond hebben opgenomen, werd in de tweede studie een oraal infectiemodel gebruikt om schapen te infecteren met *E. coli* O157:H7 **(Hoofdstuk 2)**. Een primaire orale inoculatie resulteerde in uitscheiding van *E. coli* O157:H7 in de faeces en detectie van antistoffen tegen intimine, EspA en EspB. De antistoffentiters verdwenen wanneer de uitscheiding verminderde. Een secundaire inoculatie leidde tot een langere uitscheiding, zelfs in de aanwezigheid van een booster antistoffenrespons. Cellulaire responsen volgden een gelijkaardig patroon als de antistoffen, hoewel de secundaire cellulaire respons lager was. De detectie van antigeen-specifieke antistofsecreterende cellen duidt op een systemische respons

in de milt en een lokale immuunrespons in het terminale rectum. Gecombineerd met de resultaten van de rectale inoculatiestudie suggereren deze bevindingen dat *E. coli* O157:H7 de dunne darm moet passeren om een antistoffenrespons op te wekken. Toch beschermen deze antistoffen niet tegen herinfectie.

Uitscheiding van *E. coli* O157:H7 door herkauwers kan weken, zelfs maanden aanhouden, maar stopt uiteindelijk. De derde studie werd opgezet om de rol van de immuunrespons tijdens het verdwijnen van de infectie te bepalen **(Hoofdstuk 3)**. Eerder geïnfecteerde dieren werden geherinfecteerd met *E. coli* O157:H7 en geëuthanazeerd wanneer de uitscheiding daalde om de systemische en lokale immuunrespons tegen intimine, EspA, EspB en Tir te bestuderen. Daarnaast werd de invloed van voorafgaande immunisatie met deze antigenen nagegaan. ELISPOT testen detecteerden een mucosale antistoffenrespons tegen EspA, in de jejunale Peyerse platen van gevaccineerde dieren en in de ileale Peyerse platen van niet-gevaccineerde dieren. IL-12 en IFN-γ werden geproduceerd door cellen van mesenteriale lymfeknopen, terwijl IL-10 en TNF-α ook geproduceerd werden door intestinale lymfocyten. Er werden individuele verschillen tussen langer en korter uitscheidende dieren waargenomen, wat het belang van mucosale immuunresponsen tijdens *E. coli* O157:H7 aanduidt. Nochtans lijkt infectie met *E. coli* O157:H7 niet te resulteren in de inductie van langlevende geheugencellen, en is mucosale bescherming vermoedelijk beperkt in plaats en tijd.

Het laatste deel van deze thesis bevat de algemene discussie en de belangrijkste conclusies van dit werk. De experimenten tonen aan dat *E. coli* O157:H7 schapen kan koloniseren op een langdurige manier. Passage door de dunne darm lijkt belangrijk te zijn voor het opwekken van immuunresponsen, daar serum antistoffen enkel gezien werden na orale inoculatie en niet na rectale inoculatie. Cellulaire responsen waren echter in beide gevallen aanwezig. Hoewel werd aangetoond dat cytokineresponsen optreden op het moment dat de infectiedruk lager wordt, blijft het onduidelijk welke immuunmechanismen betrokken zijn in het verdwijnen van de infectie. Verder onderzoek is noodzakelijk om de mechanismen die zorgen voor persistentie en het verdwijnen van *E. coli* O157:H7 infectie bij runderen en schapen op te helderen.

Curriculum Vitae

Curriculum Vitae

Kris Vande Walle werd op 8 december 1982 geboren te Gent. In 2000 beëindigde ze haar studies algemeen secundair onderwijs richting Latijn-wiskunde aan het Koninklijk Lyceum te Gent. Onmiddellijk daarna begon ze met de studies Biomedische Wetenschappen aan de Universiteit Gent, waar ze in 2004 afstudeerde met grote onderscheiding. Na een korte ervaring als Medical Writer bij Tibotec in Mechelen, koos ze voor het wetenschappelijk onderzoek. In 2005 werd ze aangenomen als doctoraatsstudent aan het Laboratorium voor Immunologie, waar ze onderzoek verrichte naar de interactie van *E. coli* O157:H7 met het immuunsysteem van schapen. Dit onderzoek werd gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid voor de voedselketen en Leefmilieu en werd uitgevoerd onder begeleiding van Prof. Dr. Eric Cox. In 2007 behaalde ze het FELASA certificaat categorie C in de proefdierkunde aan de Faculteit Diergeneeskunde, Universiteit Gent. Ze was betrokken bij het FOODZOON project in samenwerking met de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid en vanaf mei 2010 werkte ze aan een KMO-innovatieproject in samenwerking met VEOS, een bedrijf dat dierlijke grondstoffen verwerkt tot voedingsingrediënten. Kris nam actief deel aan internationale congressen en is auteur en co-auteur van verschillende wetenschappelijke publicaties in internationale tijdschriften.

Publications

Vande Walle K., Atef Yekta M., Verdonck F., De Zutter L., Cox E. (2010) Rectal inoculation of sheep with *E. coli* O157:H7 results in persistent infection in the absence of a protective immune response. Vet. Microbiol., in press.

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Vande Walle K., Entrican G., Wattegedera S., De Zutter L., Vanrompay D., Cox E. A preliminary investigation of the role of the immune system in clearance of *E. coli* O157:H7 infection in sheep. Manuscript in preparation.

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Joris M.-A., De Zutter L., **Vande Walle K.**, Cox E. Preliminary bacteriological and immunological screening of verocytotoxigenic Escherichia coli in slaughter cattle. Manuscript in preparation.

Participation in international conferences

Vande Walle K., Atef Yekta M., De Zutter L., Goddeeris B., Cox E., Verdonck F. Development of an *Escherichia coli* O157:H7 infection model in sheep. EADGENE *E. coli* and Salmonella workshop. 7 June 2007, Utrecht, The Netherlands (abstract and poster presentation).

Vande Walle K., De Zutter L., Goddeeris B., Cox E. Study of the immune response of sheep against *E. coli* O157:H7 in a rectal and oral inoculation model. Pathogenic *E. coli* Network Conference, Epidemiology and transmission of pathogenic *E. coli.* 25-26 September 2008, Stockholm, Sweden (abstract and poster presentation).

Vande Walle K., De Zutter L., Goddeeris B., Cox E. Comparison of immune responses of sheep against *E. coli* O157:H7 in a rectal and an oral inoculation model. 7th International symposium on Shiga toxin (Verotoxin) producing Escherichia coli infections. 10-13 May 2009, Buenos Aires, Argentina (abstract and poster presentation).

Pathogenic *E. coli* Network Conference, Control and Management of Pathogenic *E. coli.* 17-18 September 2009, Dublin, Ireland (participation).

Joris M-A., De Zutter L., Cox E., **Vande Walle K.** Preliminary immunological screening of STEC-shedding cattle. 22nd International ICFMH Symposium (Food Micro 2010): Microbial behavior in the food chain. 30 August-2 September 2010, Copenhagen, Denmark (abstract and poster presentation).

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